

NEUROTROPHIC GROWTH FACTOR

5 The present invention is concerned with a neurotrophic factor and, in particular, with cloning and expression of a novel member of the GDNF family of neurotrophic factors, designated herein as "enovin" (EVN).

Introduction

10 Neurotrophic factors are involved in neuronal differentiation, development and maintenance. These proteins can prevent degeneration and promote survival of different types of neuronal cells and are thus
15 potential therapeutic agents for neurodegenerative diseases. Glial cell-line derived neurotrophic factor (GDNF) was the first member of a growing subfamily of neurotrophic factors structurally distinct from the neurotrophins. GDNF is a member of the transforming
20 growth factor β (TGF- β) superfamily of growth factors, characterized by a specific pattern of seven highly conserved cysteine residues within the amino acid sequence (Kingsley, 1994). GDNF was originally
25 purified using an assay based on its ability to maintain the survival and function of embryonic ventral midbrain dopaminergic neurons *in vitro* (Lin et al., 1993). Other neuronal cell types in the central (CNS) or peripheral nervous systems (PNS) are also
30 responsive to the survival effects of GDNF (Henderson et al., 1994, Buj-Bello et al., 1995, Mount et al., 1995, Oppenheim et al., 1995). GDNF is produced by cells in an inactive proform, which is cleaved

specifically at a RXXR furin recognition site to
produce active (mature) GDNF (Lin et al., 1993).
Exogenous administration of GDNF has potent
neuroprotective effects in animal models of
5 Parkinson's disease, a common neurodegenerative
disorder characterised by the loss of up to 70% of
dopaminergic cells in the substantia nigra of the
brain (Beck et al., 1995, Tomac et al., 1995, Gash et
al., 1996, Choi-Lundberg et al., 1997, Bilang-Bleuel
10 et al., 1997).

Recently, additional neurotrophic factors of the
GDNF family have been discovered. Neurturin (NTN) was
purified from conditioned medium from Chinese hamster
ovary (CHO) cells using an assay based on its ability
15 to promote the survival of sympathetic neurons in
culture (Kotzbauer et al., 1996). The mature NTN
protein is 57% similar to mature GDNF. Persephin (PSP)
was discovered by cloning using degenerate primer PCR
with genomic DNA as a template. The mature PSP, like
20 mature GDNF, promotes the survival of ventral midbrain
dopaminergic neurons and of motor neurons in culture
(Milbrandt et al., 1998). The similarity of the mature
PSP protein with mature GDNF and NTN is $\approx 50\%$.
Artemin (ARTN) was discovered by DNA database
25 searching and is a survival factor of sensory and
sympathetic neurons in culture (Baloh et al, 1998b).

GDNF, NTN, PSP and ARTN require a heterodimeric
receptor complex in order to carry out downstream
intracellular signal transduction. GDNF binds to the
30 GDNF family receptor alpha 1 (GFR α -1; GFR α
Nomenclature Committee, 1997) subunit, a glycosyl-
phosphatidyl-inositol (glycosyl-PtdIns) anchored
membrane protein (Jing et al., 1996, Treanor et al.,

1996, Sanicola et al., 1997). The GDNF/GFR α -1 complex subsequently binds to and activates the cRET proto-oncogene, a membrane bound tyrosine kinase (Durbec et al., 1996, Trupp et al., 1996), resulting in the phosphorylation of tyrosine residues in cRET and subsequent activation of downstream signal transduction pathways (Worby et al., 1996). Several other members of the GFR α family of ligand binding receptors have been characterised (Baloh et al., 1997, Sanicola et al., 1997, Klein et al., 1997, Buj-Bello et al., 1997, Suvanto et al., 1997). GFR α -2 and GFR α -3 (Jing et al., 1997, Masure et al., 1998, Woby et al., 1998, Naveilham et al., 1998, Baloh et al., 1998a) have been identified by a number of different groups. GFR α -1 and GFR α -2 are widely expressed in almost all tissues and expression may be developmentally regulated (Sanicola et al., 1997, Widenfalk et al., 1997).

GFR α -3 is not expressed in the developing or adult central nervous system, but is highly expressed in several developing and adult sensory and sympathetic ganglia of the peripheral nervous system (Widenfalk et al., 1998, Naveilhan et al., 1998, Baloh et al., 1998a). A fourth family member, GFR α -4, was cloned from chicken cDNA (Thompson et al., 1998). GFR α -1 is the preferred receptor for GDNF, whereas GFR α -2 preferentially binds NTN (Jing et al., 1996, Treanor et al., 1996, Klein et al., 1997). Chicken GFR α -4 forms a functional receptor complex for PSP in combination with cRET (Enokido et al., 1998). Cells expressing both GFR α -3 and cRET were shown not to respond to either GDNF, NTN or PSP (Worby et al., 1998, Baloh et al., 1998a). Recently, ART has been

shown to signal through cRET using GFR α -3 as the preferred ligand-binding receptor (Baloh et al., 1998b). Cross-talk between the neurotrophic factors and GFR α receptors is possible *in vitro*, as GDNF can
5 bind to GFR α -2 or GFR α -3 in the presence of cRET (Sanicola et al., 1997, Trupp et al., 1998) and NTN can bind to GFR α -1 with low affinity (Klein et al., 1997). In summary, GDNF, NTN, PSP and ART are part of a neurotrophic signalling system whereby different
10 ligand-binding subunits (GFR α -1 to -4) can interact with the same Tyrosine kinase subunit (cRET). The physiological relevance of these *in vitro* findings was recently shown in gene knockout studies (reviewed by Rosenthal, 1999), which clearly show that GDNF
15 interacts with GFR α -1 *in vivo*, whereas NTN is the preferred ligand for GFR α -2.

The present inventors have identified, cloned, expressed, chromosomally localized and characterized Enovin (EVN), the fourth member of the GDNF family.
20 The knowledge of the mature EVN protein has been extended with the discovery of different functional and non-functional mRNA splice variants. Moreover, we present expression data, binding data of EVN to GFR α -3 and *in vitro* effects of EVN on neurite outgrowth and
25 protection against taxol-induced neurotoxicity in staurosporine-differentiated SH-SY5Y human neuroblastoma cell cultures.

Summary of the Invention

30 In the present application, there is provided a nucleic acid molecule encoding a novel human

neurotrophic growth factor, "enovin", an expression
vector comprising said nucleic acid molecule, a host
cell transformed with said vector, a neurotrophic
growth factor encoded by said nucleic acid molecule,
5 isolated enovin, compounds which act as agonists or
antagonists of enovin and pharmaceutical compositions
containing the nucleic acid or the enovin protein or
the agonists or antagonists thereof.

10 **Detailed Description of the Invention**

According to a first aspect of the present
invention there is provided a nucleic acid molecule
encoding a human neurotrophic growth factor,
15 designated herein as enovin, having the amino acid
sequence illustrated in Figure 21, or encoding a
functional equivalent, derivative or bioprecursor of
said growth factor. Preferably, said nucleic acid
molecule is DNA and even more preferably a cDNA
20 molecule.

Preferably, the nucleic acid according to the
invention comprises the sequence from positions 81 to
419 of the sequence illustrated in Figure 1 and more
preferably from positions 81 to 422 and even more
25 preferably the complete sequence illustrated in Figure
1.

The nucleic acid molecule from position 81 to 419
is believed to encode the sequence of the mature
enovin protein subsequent to processing of the proform
30 of the protein at the RXXR processing site present in
the stable proform of said enovin protein.

There is also provided by the invention an
antisense molecule capable of hybridising to any of

the nucleic acid sequences according to the invention, under high stringency conditions, which would be well known to those skilled in the art.

Stringency of hybridisation as used herein refers
5 to conditions under which polynucleic acids are stable. The stability of hybrids is reflected in the melting temperature (T_m) of the hybrids. T_m can be approximated by the formula:

10
$$81.5^{\circ}\text{C} - 16.6 (\log_{10}[\text{Na}^+] + 0.41 (\% \text{G\&C}) - 600/l$$

wherein l is the length of the hybrids in nucleotides. T_m decreases approximately by 1-1.5°C with every 1% decrease in sequence homology.

15 Advantageously, the nucleic acid molecule according to the invention may be used to express the human neurotrophic growth factor according to the invention, in a host cell or the like using an appropriate expression vector.

20 An expression vector according to the invention includes vectors capable of expressing DNA operatively linked to regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments.

25 Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector may include a promoter such as the lac promoter and
30 for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector may include a heterologous or homologous promoter for RNA polymerase

II, a downstream polyadenylation signal, the start
codon AUG, and a termination codon for detachment of
the ribosome. Such vectors may be obtained
commercially or assembled from the sequences described
5 by methods well known in the art.

Thus, an expression vector refers to a
recombinant DNA or RNA construct, such as a plasmid, a
phage, recombinant virus or other vector that upon
introduction into an appropriate host cell results in
10 expression of the DNA or RNA fragments. Appropriate
expression vectors are well known to those skilled in
the art and include those that are replicable in
eukaryotic cells and/or prokaryotic cells and those
that remain episomal or those which integrate into the
15 host cell genome.

The antisense molecule capable of hybridising to
the nucleic acid according to the invention may be
used as a probe or as a medicament or in a
pharmaceutical composition.

20 Nucleic acid molecules according to the invention
may be inserted into the vectors described in an
antisense orientation in order to provide for the
production of antisense RNA. Antisense RNA or other
antisense nucleic acids may be produced by synthetic
25 means.

A further aspect of the invention comprises the
host cell transformed, transfected or infected with
the expression vector according to the invention,
which cell preferably comprises a eukaryotic cell and
30 more preferably a mammalian cell.

Incorporation of cloned DNA into a suitable
expression vector for subsequent transformation of
said cell and subsequent selection of the transformed

cells is well known to those skilled in the art as provided in Sambrook et al (1989) Molecular Cloning, A Laboratory manual, Cold Spring Harbour Laboratory Press.

5 A further aspect of the present invention comprises a nucleic acid molecule having at least 15 nucleotides of the nucleic acid molecule according to the invention and preferably from 15 to 50 nucleotides.

10 These sequences may, advantageously be used as probes or primers to initiate replication or the like. Such nucleic acid molecules may be produced according to techniques well known in the art, such as by recombinant or synthetic means. They may also be used
15 in diagnostic kits or devices or the like for detecting for the presence of a nucleic acid according to the invention. These tests generally comprise contacting the probe with a sample under hybridising conditions and detecting for the presence of any
20 duplex formation between the probe and any nucleic acid in the sample.

 According to the present invention these probes may be anchored to a solid support. Preferably, they are present on an array so that multiple probes can
25 simultaneously hybridize to a single biological sample. The probes can be spotted onto the array or synthesised *in situ* on the array. (See Lockhart et al., Nature Biotechnology, vol. 14, December 1996 "Expression monitoring by hybridisation into high
30 density oligonucleotide arrays". A single array can contain more than 100, 500 or even 1,000 different probes in discrete locations.

 Nucleic acid molecules according to the invention

may also be produced using such recombinant or synthetic means, such as, for example, using PCR cloning mechanisms which generally involve making a pair of primers, which may be from approximately 10 to 50 nucleotides to a region of the gene which is desired to be cloned, bringing the primers into contact with mRNA, cDNA, or genomic DNA from a human cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified region or fragment and recovering the amplified DNA. Generally, such techniques as defined herein are well known in the art, such as described in Sambrook et al (Molecular Cloning: a Laboratory Manual, 1989).

The nucleic acids or oligonucleotides according to the invention may carry a revealing label. Suitable labels include radioisotopes such as ^{32}P or ^{35}S , enzyme labels or other protein labels such as biotin or fluorescent markers. Such labels may be added to the nucleic acids or oligonucleotides of the invention and may be detected using known techniques per se.

Advantageously, human allelic variants or polymorphisms of the DNA molecule according to the invention may be identified by, for example, probing cDNA or genomic libraries from a range of individuals for example from different populations. Furthermore, nucleic acids and probes according to the invention may be used to sequence genomic DNA from patients using techniques well known in the art, such as the Sanger Dideoxy chain termination method, which may advantageously ascertain any predisposition of a patient to certain disorders associated with a growth

factor according to the invention.

Further provided by the present invention is a transgenic cell, tissue or organism comprising a transgene capable of expressing the human neurotrophic factor enovin according to the invention.

The term "transgene capable of expression" as used herein means any suitable nucleic acid sequence which leads to expression of a neurotrophic factor having the same function and/or activity of a neurotrophic factor according to the invention. The transgene may include, for example, genomic nucleic acid isolated from human cells or synthetic nucleic acid including cDNA, integrated into the chromosome or in an extrachromosomal state.

Preferably, the transgene comprises a vector according to the invention, which vector includes a nucleic acid molecule encoding said neurotrophic factor, or a functional fragment of said nucleic acid molecule. A "functional fragment" of said nucleic acid should be taken to mean a fragment of the gene or cDNA encoding said neurotrophic factor or a functional equivalent thereof, which fragment is capable of being expressed to produce a functional neurotrophic growth factor according to the invention. Thus, for example, fragments of the neurotrophic growth factor according to the invention which correspond to the specific amino acid residues interacting with the corresponding receptor also form part of the present invention and which fragments may serve to function as agonists activating the corresponding receptor of the growth factor according to the invention so as to elicit its growth promoting and survival sustaining effects on cells. This aspect of the invention also includes

differentially spliced isoforms and transcriptional starts of the nucleic acids according to the invention.

5 In accordance with the present invention, a defined nucleic acid includes not only the identical nucleic acid but also any minor base variations including in particular, substitutions in bases which result in a synonymous codon (a different codon specifying the same amino acid residue) due to the
10 degenerate code in conservative amino acid substitutions. The term "nucleic acid molecule" also includes the complementary sequence to any single stranded sequence given regarding base variations.

15 According to a further aspect the invention provides an isolated human neurotrophic growth factor, encoded by a nucleic acid molecule as defined herein. Preferably, the growth factor comprises an amino acid sequence from position 27 to 139 of the amino acid sequence of Figure 1 or a functional equivalent,
20 derivative or bioprecursor thereof.

A "functional equivalent" as defined herein should be taken to mean a growth factor that exhibits all of the growth properties and functionality associated with the growth factor enovin. A
25 "derivative" of enovin as defined herein is intended to include a polypeptide in which certain amino acids have been altered or deleted or replaced with other amino acids and which polypeptide retains the biological activity of enovin and/or which polypeptide
30 can react with antibodies raised using enovin according to the invention as the challenging antigen.

Encompassed within the scope of the present invention are hybrid and modified forms of enovin,

including fusion proteins and fragments. The hybrid and modified forms include, for example, when certain amino acids have been subjected to some modification or replacement, such as for example, by point mutation yet which modifications still result in a protein which retains the biological activity of enovin, according to the invention. Specific nucleic acid sequences can be altered by those of skill in the art to produce a growth factor exhibiting the same or substantially properties to enovin.

As is well known in the art, many proteins are produced in vivo with a (pre) signal sequence at the N-terminus of the protein. Furthermore, such proteins may comprise a further pro sequence that represents a stable precursor to the mature protein. Such pre and pro sequences are not generally necessary for biological activity. The enovin molecule according to the invention includes not only the full length sequence illustrated in Figure 21 but from position 27 to 139, which follows the RXXR proteolytic processing site present in growth factors of this type and which is believed to represent the mature sequence of enovin.

A defined protein, polypeptide or amino acid sequence according to the invention includes not only the identical amino acid sequence but isomers thereof in addition to minor amino acid variations from the natural amino acid sequence including conservative amino acid replacements (a replacement by an amino acid that is related in its side chains). Also included are amino acid sequences which vary from the natural amino acid but result in a polypeptide which is immunologically identical or similar to the

polypeptide encoded by the naturally occurring sequence.

Proteins or polypeptides according to the invention further include variants of such sequences, including naturally occurring allelic variants which are substantially homologous to said proteins or polypeptides. In this context, substantial homology is regarded as a sequence which has at least 70%, and preferably 80%, 90% or 95% amino acid homology with the proteins or polypeptides encoded by the nucleic acid molecules according to the invention.

Neurotrophic growth factors expressed by the host cells according to the invention are also encompassed within the present invention.

The present invention is further directed to inhibiting the neurotrophic growth factor according to the invention *in vivo* by the use of antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the part of the DNA sequence coding for the mature protein of the present invention is used to design an antisense RNA oligonucleotide of from 10 to 50 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple-helix - see Lee et al. Nucl. Acids. Res., 6:3073 (1979); Cooney et al., Science, 241:456 (1988); and Dervan et al., Science, 251: 1360 (1991), thereby preventing transcription and the production of enovin. The antisense RNA oligonucleotide hybridises to the mRNA *in vivo* and

blocks translation of an mRNA molecule into enovin.

Because of the sequence similarity between the growth factor described herein with previously identified growth factors of the GDNF family, enovin
5 is also believed to be capable of promoting cell survival and growth and in treating disorders resulting from defects in function or expression of said neurotrophic factor.

The nucleic acid molecules or the neurotrophic
10 factor according to the invention may, advantageously, therefore be used to treat or prevent neural disorders in a subject by administering to said subject an amount of a nucleic acid molecule or growth factor according to the invention in sufficient concentration
15 to reduce the symptoms of said disorder. Thus, the nucleic acid molecules of the invention may be used to promote maintenance and survival of neuronal cells and for treating neuronal disorders or neurodegenerative conditions including Parkinson's disease, Alzheimer's
20 disease, peripheral neuropathy, amyotrophic lateral sclerosis, peripheral and central nerve trauma or injury and exposure to neurotoxins.

The neurotrophic growth factor according to the invention has, advantageously, been observed to confer
25 a neurotrophic or neuroprotective effect on neuronal cells or cell populations, particularly those neuronal cells or cell populations which have been induced to undergo apoptosis. Accordingly, the nucleic acid or the enovin growth factor itself according to the
30 invention may additionally be used in treating neurodegenerative disorders such as stroke, Huntingtons disease, peripheral neuropathy, acute brain injury, nervous system tumours, multiple

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an epitope thereof and recovering immune serum.

Monoclonal antibodies may be prepared according to

known techniques such as described by Kohler R. and

Milstein C., Nature (1975) 256, 495-497.

5 Antibodies according to the invention may,

advantageously, be used in a method of detecting for

the presence of a growth factor according to the

invention, which method comprises reacting the

antibody with a sample and identifying any protein

10 bound to said antibody. A kit is also provided for

performing said method which comprises an antibody

according to the invention and means for reacting the

antibody with said sample.

Also provided by the present invention is a kit

or device for detecting for the presence of a

neurotrophic growth factor according to the invention

in a sample, comprising an antibody as described above

and means for reacting said antibody and said sample.

Proteins which interact with the neurotrophic

factor of the invention, such as for example it's

corresponding cellular receptor may be identified by

investigating protein-protein interactions using the

two-hybrid vector system which is well known to

molecular biologists (Fields & Song, Nature 340:245

25 1989). This technique is based on functional

reconstitution in vivo of a transcription factor which

activates a reporter gene. More particularly the

technique comprises providing an appropriate host cell

with a DNA construct comprising a reporter gene under

30 the control of a promoter regulated by a transcription

factor having a DNA binding domain and an activating

domain, expressing in the host cell a first hybrid DNA

sequence encoding a first fusion of a fragment or all

of a nucleic acid sequence according to the invention
and either said DNA binding domain or said activating
domain of the transcription factor, expressing in the
host at least one second hybrid DNA sequence, such as
5 a library or the like, encoding putative binding
proteins to be investigated together with the DNA
binding or activating domain of the transcription
factor which is not incorporated in the first fusion;
detecting any binding of the proteins to be
10 investigated with a protein according to the invention
by detecting for the presence of any reporter gene
product in the host cell; optionally isolating second
hybrid DNA sequences encoding the binding protein.

An example of such a technique utilises the GAL4
15 protein in yeast. GAL4 is a transcriptional activator
of galactose metabolism in yeast and has a separate
domain for binding to activators upstream of the
galactose metabolising genes as well as a protein
binding domain. Nucleotide vectors may be constructed,
20 one of which comprises the nucleotide residues
encoding the DNA binding domain of GAL4. These binding
domain residues may be fused to a known protein
encoding sequence, such as for example the nucleic
acids according to the invention. The other vector
25 comprises the residues encoding the protein binding
domain of GAL4. These residues are fused to residues
encoding a test protein, preferably from the signal
transduction pathway of the vertebrate in question.
Any interaction between neurotrophic factor encoded by
30 the nucleic acid according to the invention and the
protein to be tested leads to transcriptional
activation of a reporter molecule in a GAL-4
transcription deficient yeast cell into which the

vectors have been transformed. Preferably, a reporter molecule such as β -galactosidase is activated upon restoration of transcription of the yeast galactose metabolism genes.

- 5 The receptor for enovin has been identified by the present inventors as GFR α 3. Assays may therefore be prepared to identify agonist or antagonistic compounds of enovin. This assay may also be used in association with other neurotrophic growth factors and their corresponding receptors. Compounds identified may be used to treat or prevent disorders such as Parkinson's disease, Alzheimer's disease, neuronal disorders associated with expanded polyglutamine sequences, such as, Huntingdon's disease, peripheral neuropathy, acute brain injury, nervous system tumours, multiple sclerosis, amyotrophic lateral sclerosis, peripheral nerve trauma or injury exposure to neurotoxins, multiple endocrine neoplasia and familial Hirschsprung disease, Prion associated diseases, Creutzfeld - Jacob disease, stroke, pain syndromes with a substantially peripheral or central neurogenic component, rheumatic/inflammatory diseases as well as conductance disturbances by administering to an individual an amount of said agonist or antagonist in sufficient concentration to prevent or treat said neural disorders. Such compounds may also be included in pharmaceutical compositions together with a pharmaceutically acceptable carrier, diluent or excipient therefor.
- 10
- 15
- 20
- 25
- 30 Agonists or antagonists of a growth factor (such as for example enovin) may be identified in one embodiment by contacting a cell tissue or organism expressing an appropriate receptor and cRET with a

5 An alternative embodiment of the invention
comprises a method of identifying agonists or
antagonists of a neurotrophic growth factor said
method comprising contacting a cell tissue or organism
expressing an appropriate receptor of said growth
0 factor and cRET with a candidate compound in the
presence of said growth factor, measuring the level of
activation of a signalling kinase in the signal
transduction pathway of which said appropriate
receptor is a component following addition of an
5 antibody specific for said signal kinase conjugated to
a reporter molecule compared to a cell tissue or
organism which has not been contacted with said
compound.

25 The compounds identified in the assays of the present invention may advantageously be used to enhance the gastrointestinal motility and therefore may be useful in treating conditions related to a hampered or impaired gastrointestinal transit.

Accordingly, such compounds may be useful in
30 treating warm-blooded animals, including humans,
suffering from conditions related to a hampered or
impaired gastric emptying or more generally suffering
from conditions related to a hampered or impaired

gastrointestinal transit. Consequently a method of treatment is provided for relieving patients from conditions, such as, for example, gastrooesophageal reflux, dyspepsia, gastroparesis, post-operative
5 ileus, and intestinal pseudo-obstruction.

Dyspepsia is an impairment of the function of digestion, that can arise as a symptom of a primary gastrointestinal dysfunction, especially a gastrointestinal dysfunction related to an increased
10 muscle tone or as a complication due to other disorders such as appendicitis, galbladder disturbances, or malnutrition. Dyspeptic symptoms are for example a lack of appetite, feeling of fullness, early satiety, nausea, vomiting and bloating.

Gastroparesis can be brought about by an
15 abnormmaly in the stomach or as a complication of diseases such as diabetes, progressive systemic sclerosis, anorexia, nervosa and myotonic dystrophy.

Post-operative ileus is an obstruction or a
20 kinetic impairment in the intestine due to a disruption in muscle tone following surgery.

Intestinal pseudo-obstruction is a condition characterized by constipation, colicky pain, and vomiting, but without evidence of physical
25 obstruction.

The compounds of the present invention can thus be used either to take away the actual cause of the condition or to alleviate the symptoms of the conditions.

30 Additionally some of the compounds being stimulators of kinetic activity on the colon, may be useful to normalize or to improve the intestinal transit in subjects suffering from symptoms related to

disturbed motility, e.g. a decreased peristalsis of the small and large intestine alone or in combination with delayed gastric emptying.

5 In view of the colon kinetic utility of the compounds of the present invention, there is provided a method of treating warm-blooded animals, including humans, suffering from motility disorders of the intestinal system, such as, for example, constipation, pseudo-obstruction, intestinal atony, post-operative
10 intestinal atony, irritable bowel syndrome (IBS), and drug-induced delayed transit.

Compounds identified as antagonists according to the assays of the present invention may also be of potential use in the treatment or prophylaxis of
15 gastrointestinal conditions resulting from increased peristaltic movements in the intestines such as diarrhea (including secretory diarrhea, bacterial induced diarrhea, choleic diarrhea, traveller's diarrhea and psychogenic diarrhea), Crohn's disease,
20 spastic colon, irritable bowel syndrome (IBS), diarrheapredominant irritable bowel gastrointestinal hypersensitivity.

In view of the utility of the compounds of the invention, it follows that the present invention also
25 provides a method of treating warm-blooded animals, including humans suffering from gastrointestinal conditions such as irritable bowel syndrome (IBS), especially the diarrhoea aspects of IBS. Consequently a method of treatment is provided for relieving
30 patients suffering from conditions such as irritable bowel syndrom (IBS), diarrheapredonminant irritable bowel syndrome, bowel hypersensitivity, and the reduction of pain associated with gastrointestinal

hypersensitivity.

The present compounds may also be of potential use in other gastrointestinal disorders, such as those associated with upper gut motility, and as antiemetics for treating emesis, and cytotoxic drug and radiation induced emesis.

Inflammatory bowel diseases include, for example, ulcerative colitis, Crohn's disease and the like.

A further aspect of the invention also comprises a method of treating a disorder mediated by expression of enovin according to the invention by administering to a patient an amount of an antisense molecule or an antagonist thereof according to the invention in sufficient concentration to alleviate or reduce the symptoms of said disorder.

Disorders mediated by inactivation or inhibiting expression of enovin may also advantageously be treated by administering to an individual an amount of a compound identified as an agonist of enovin in sufficient concentration to reduce or prevent the symptoms of the disorder.

In a further aspect, the invention provides a method for making a pharmaceutical formulation for the treatment of diseases associated with human neurotrophic growth factor enovin, said method comprising, selecting a candidate compound identified as an agonist or antagonist of enovin according to the invention, manufacturing bulk quantities of said compound and formulating the compound manufactured in a pharmaceutically acceptable carrier.

As will be seen in more detail from the examples below, enovin has been successful in reducing taxol induced sensory deficits. Enovin may therefore play a

possible role in pain syndromes with a substantially peripheral and central neurogenic component, rheumatic diseases as well as conductance disturbances and can play a modulatory role in sensory processes after

5 transdermal, topical, local central (such as epidural, intrathecal, ICV, intraplexus, intraneuronal) per oral, rectal and systemic application. Therefore, in the same manner as described herein for other conditions mediated by enovin, these conditions may be

10 alleviated or even prevented by administering either an antisense molecule, a nucleic acid, enovin protein, pharmaceutical composition, or a compound identified as an agonist or an antagonist, as appropriate, according to the invention, in sufficient

15 concentrations to alleviate or prevent the symptoms of said disorder(s).

The therapeutic or pharmaceutical compositions of the present invention can be administered by any suitable route known in the art including for example

20 intravenous, subcutaneous, intramuscular, transdermal, intrathecal or intracerebral or administration to cells in ex vivo treatment protocols. Administration can be either rapid as by injection or over a period of time as by slow infusion or administration of slow

25 release formulation. For treating tissues in the central nervous system, administration can be by injection or infusion into the cerebrospinal fluid (CSF).

Enovin can also be linked or conjugated with

30 agents that provide desirable pharmaceutical or pharmacodynamic properties. For example, it can be coupled to any substance known in the art to promote penetration or transport across the blood-brain

barrier such as an antibody to the transferrin receptor, and administered by intravenous injection.

Enovin, the antisense molecules or indeed the compounds identified as agonists or antagonists of enovin according to the invention may be used in the form of a pharmaceutical composition, which may be prepared according to procedures well known in the art. Preferred compositions include a pharmaceutically acceptable vehicle or diluent or excipient, such as for example, a physiological saline solution. Other pharmaceutically acceptable carriers including other non-toxic salts, sterile water or the like may also be used. A suitable buffer may also be present allowing the compositions to be lyophilized and stored in sterile conditions prior to reconstitution by the addition of sterile water for subsequent administration. Incorporation of enovin into a solid or semi-solid biologically compatible matrix may be carried out which can be implanted into tissues requiring treatment.

The carrier can also contain other pharmaceutically acceptable excipients for modifying other conditions such as pH, osmolarity, viscosity, sterility, lipophilicity, solubility or the like. Pharmaceutically acceptable excipients which permit sustained or delayed release following administration may also be included.

The enovin protein or the nucleic acid molecules or compounds according to the invention may be administered orally. In this embodiment they may be encapsulated and combined with suitable carriers in solid dosage forms which would be well known to those skilled in the art.

As would be well known to those of skill in the art, the specific dosage regime may be calculated according to the body surface area of the patient or the volume of body space to be occupied, dependent upon the particular route of administration to be used. The amount of the composition actually administered will, however, be determined by a medical practitioner, based on the circumstances pertaining to the disorder to be treated, such as the severity of the symptoms, the composition to be administered, the age, weight, and response of the individual patient and the chosen route of administration.

The present invention may be more clearly understood by the following examples which are purely exemplary and by reference to the accompanying drawings wherein:

Figure 1: is partial cDNA sequence of a neurotrophic factor according to the invention designated as enovin. The consensus sequence was obtained by PCR amplification with primers PNHsp3 and PNHapl on different cDNAs and on genomic DNA followed by cloning and sequence analysis and comparison of the obtained sequences. The predicted one letter code amino acid sequence is shown above the DNA sequence. The nucleotide residue number is shown on the right of the DNA sequence, whereas the amino acid residue number is shown to the right of the translated protein sequence. The putative RXXR cleavage site for the prodomain is indicated in bold and underlined. The putative start of the mature protein is indicated by an arrow. The seven conserved cysteine residues characteristics for all members of the TGF- β family

are indicated in bold. A potential N-glycosylation site is double underlined,

5 Figure 2: is alignment of the predicted mature protein sequences of human GDNF, NTN, PSP and EVN. The sequences were aligned using the ClustalW alignment program. Amino acid residues conserved between all three proteins are included in the black areas. Residues conserved between two or three of the sequences are shaded in grey. The 7 conserved cysteine residues characteristic for members of the TGF- β family are indicated by asterisks above the sequence. Amino acid residues are numbered to the right. The dashes indicate gaps introduced into the sequence to optimize the alignment,

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20 Figure 3: is partial cDNA sequence of enovin. The consensus sequence was obtained by PCR amplification (primary PCR with primers PNHsp1 and PNHap1 and nested PCR with primers PNHsp2 and PNHap2) on different cDNAs followed by cloning and sequence analysis and comparison of the obtained sequences. The translated one letter code amino acid sequence of nucleotides 30 to 284 (reading frame A) is shown above the sequence and numbered to the right (A1 to A85). This reading frame contains a putative ATG translation start codon. The translated one letter code amino acid sequence of nucleotides 334 to 810 (reading frame B) is shown above the sequence and numbered to the right (B1 to B159). This reading frame contains the region of homology with GDNF, NTN and PSP. The nucleotide residue number is shown to the right of the DNA sequence. The putative RXXR cleavage site for the

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prodomain is indicated in bold and underlined. The putative start of the mature protein is indicated by an arrow. The seven conserved cysteine residues characteristic for all members of the TGF- β family are indicated in bold. A potential N-glycosylation site is double underlined,

Figure 4: is an illustration of the chromosomal localisation of human Enovin. (A) Diagram of FISH mapping results for Enovin. Each dot represents the double FISH signals detected on human chromosome 1, region p31.3-p32. (B) Example of FISH mapping of Enovin. The left panel shows the FISH signals on chromosome 1. The right panel shows the same mitotic figure stained with 4',6-diamidino-2-phenylindole to identify chromosome 1,

Figure 5. is an illustration of expression of Enovin in different human tissues. (A), (B), (C) Northern blot analysis of tissue expression of Enovin. The expression of Enovin mRNA in different human tissues was assessed using a probe corresponding to part of the coding region of Enovin (including the region coding for the mature Enovin protein) to analyse blots of human poly(A) rich RNA. (A) Multiple Tissue Northern (MTN) blot; (B) MTN blot II) Fetal MTN blot II. Panel (D) shows an autoradiography of the human RNA master blot probed with the same Enovin cDNA fragment. Panel (E) shows the location of human tissue mRNA samples on the RNA master blot from (D),

Figure 6: is a graphic illustration of the total survival of SH-SY5Y cells after 72 hours treatment

with 10⁻⁶M taxol and the effect of increasing doses of enovin on this survival, normalised to the condition of solvent. SH-SY5Y cells are differentiated for 5 days with 25nM staurosporine before application of taxol. Data are from two independent experiments in sixtuplate. Mean and st. dev. is shown,

Figure 7: is a graphic representation of the effect of increasing concentrations of enovin over 48 hours on neurite outgrowth of staurosporine - differentiated SH-SY5Y cells, normalised to the condition of solvent. SH-SY5Y cells are differentiated for 5 days with 25nM staurosporine before starting the 48 hour experiment. As a positive control, the differentiating effect of 25nM staurosporine is shown. Neurite length is calculated on at least 5000 cells. Data is provided from the experiments performed in duplicate. Mean and st. dev. is shown.

Figures 8 to 18: are graphic representations of the effect of enovin on proliferation of various cell types.

Figure 19: is a graphic representation of the effects of enovin on taxol-induced sensory deficits using the pin prick test. Given are the average (\pm 1 SEM) cumulative scores over time of rats treated with either 2 different doses of enovin (23 or 130 μ g/ml; n = 10 rats/ group) or vehicle / saline (n = 20 rats) after taxol. Enovin or saline / vehicle were injected in a volume of 75 μ l in the subplantar area of the right hind paw.

Figure 20: is a graphic representation of the effects of enovin on taxol-induced sensory deficits using the pin prick test. Given are the average (± 1 SEM) cumulative scores over time of rats treated with either 2 different doses of enovin (23 or 130 $\mu\text{g/ml}$; $n = 10$ rats/ group) or vehicle / saline ($n = 20$ rats) before taxol. Enovin or saline / vehicle were injected in a volume of 75 μl in the subplantar area of the right hind paw.

Figure 21: is a DNA sequence of enovin. The consensus sequence was obtained by amplification with PCR using primers PNHsp5 and PNHapl on human frontal cortex cDNA and on human genomic DNA followed by cloning, sequence analysis and comparison of the resultant sequences. The predicted amino acid sequence is shown above the DNA sequence for the only splice variant yielding a functional Enovin protein after translation. The nucleotide residue number is shown to the left of the DNA sequence, whereas the amino acid residue number is shown to the right of the translated protein sequence. 5' and 3' splice sites detected by comparison of sequenced cDNA fragments with the genomic sequence are indicated by vertical lines bending to the left or right, respectively, and are numbered consecutively. The putative RXXR furin cleavage site for the prodomain is indicated in bold and underlined. The putative start of the mature protein is indicated by an arrow. The seven conserved cysteine residues characteristic for all members of the TGF- β family are indicated in bold. A potential N-linked glycosylation site is double underlined. The 5' and 3' splice sites are numbered and encircled.

Figure 22: is an illustration of expression of different Enovin splice variants in human tissues. (A) schematic diagram of Enovin splice variants identified by RT-PCR experiments with Enovin specific primers on RNA derived from different human tissues followed by cloning and sequence analysis of PCR products. The top line shows a scale (in bp). The second line represents the Enovin genomic sequence. The position of the translation start and stop codon, of the start of the mature Enovin coding sequence and of the 5' and 3' splice sites (see Figure 21) are indicated. The right part of the figure shows the PCR products obtained by RT-PCR on ovary and on frontal cortex RNA together with a 100 bp DNA ladder. The position of the different mRNA variants is indicated together with their size (from start to stop codon). The translated proteins are shown on the left hand side. Boxes delineate regions represented in the cDNA. Dashed lines represent spliced out genomic DNA. The shaded region represents the mature Enovin coding sequence. The dotted line marks the start of the mature Enovin coding sequence. The two transcripts capable of yielding functional Enovin protein are indicated by an asterisk at the left hand side. (B) Tissue distribution of the main splice variants. The photograph shows the PCR fragments obtained by RT-PCR with Enovin specific primers on different human cDNAs. The 4 main splice variants (A to D) are indicated by arrows at the left hand side. Sizes are indicated on the right hand side based on the 100 bp DNA ladder used as size reference on the gel.

Figure 23: Predicted protein sequence of the long

splice variant of Enovin, obtained by splicing out the two introns from the DNA sequence of Figure 21.

Splice sites 5'-1 and 3'-1 are used to remove the first intron and splice sites 5'-2 and 3'-3 are used to remove the second intron. This results in a cDNA sequence having an open reading frame coding for the 228 amino acid residue protein shown above.

Figure 24: Predicted protein sequence of an alternative (short) splice variant of Enovin, obtained by splicing out the two introns from the DNA sequence of Figure 21. Splice sites 5'-1 and 3'-2 are used to remove the first intron and splice sites 5'-2 and 3'-3 are used to remove the second intron. This results in a cDNA sequence having an open reading frame coding for the 220 amino acid residue protein shown above. This protein sequence misses 8 amino acid residues compared to the sequence of Figure 23.

Figure 25: is a graphic representation of the results obtained from experiments designed to compare the levels of expression of enovin in normal diseased tissue. Enovin and GAPDH expression is represented in brain tissue, in respect of multiple sclerosis and Alzheimer's disease.

Figure 26: is a graphic representation of the results obtained to detect levels of expression of enovin and GAPDH in Parkinson's disease and cancer.

Deposits

Plasmid EVNmat/pRSETB including the DNA sequence

encoding enovin, was deposited on 6 May 1999 under
Accession No. LMBP3931, at the Belgian Coordinated
Collections of Micro-Biologie (BCCM) at Laboratorium
voor Moleculaire - Plasmidencollectie (LMBP) B9000,
5 Ghent, Belgium, in accordance with the provisions of
the Budapest Treaty of 28 April 1997.

Materials and methods

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Materials

Native Taq polymerase, ampicillin, IPTG
(isopropyl- β -D-thiogalactoside), X-gal (5-bromo-4-
chloro-3-indolyl- β -D-galactopyranoside) and all
15 restriction enzymes used were from Boehringer Mannheim
(Mannheim, Germany). 10 mM dNTP mix was purchased from
Life Technologies (Gaithersburg, MD, USA). The TOPO-TA
cloning kit was purchased from Invitrogen BV (Leek,
The Netherlands). The Qiagen plasmid mini- or midi-DNA
20 purification kit, the Qiaprep Spin Miniprep kit and
the Qiaquick gel extraction kit were purchased from
Qiagen GmbH (Dusseldorf, Germany). cDNA libraries,
MarathonTM Ready cDNA kits, human multiple tissue cDNA
(MTCTM) panels I and II multiple tissues northern
25 blots and the Advantage-GC cDNA PCR kit were obtained
from Clontech Laboratories (Palo Alto, CA, USA). All
PCR reactions were performed in a GeneAmp PCR system
9600 cyclor (Perkin Elmer, Foster City, CA, USA). LB
(Luria-Bertani) medium consists of 10 g/l of tryptone,
30 5 g/l of yeast extract and 10 g/l of NaCl. 2x
YT/ampicillin plates consist of 16 g/l of tryptone, 10
g/l of yeast extract, 5 g/l of NaCl, 15 g/l of agar

and 100 mg/l of ampicillin.

**Database homology searching and sequence
comparison.**

5

Using the complete human glial cell-line derived
neurotrophic factor (GDNF; accession no. Q99748),
neurturin (NTN; accession no. P39905) and persephin
(PSP; accession no. AF040962) cDNA derived protein
sequences as query sequences, a BLAST (Basic Local
Alignment Search Tool; Altschul et al., 1990) search
was performed on the daily update of the EMBL/GenBank
human expressed sequence tag (EST) and genomic
databases.

15

Additional BLAST searches were performed using
the genomic sequence with accession no. AC005038 and
several ESTs present in the GenBank database and
showing homology to this genomic sequence were
detected.

20

The percentage identity and percentage similarity
between members of the GDNF family was calculated
bypairwise comparison of the sequences using the
BESTFIT program (Genetics Computer Group sequence
analysis software package, version 8.0, University of
Wisconsin, Madison, WI, USA). Alignments of DNA or
protein sequences were done with the ClustalW
alignment program (EMBL, Heidelberg, Germany).

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**Oligonucleotide synthesis for PCR and
DNA sequencing.**

All oligonucleotide primers were ordered from

Eurogentec (Seraing, Belgium). Insert-specific sequencing primers (15- and 16-mers) and primers for use in PCR reactions were designed manually. DNA was prepared on Qiagen-tip-20 or -100 anion exchange or
5 Qiaquick spin columns (Qiagen GmbH, Dusseldorf, Germany) and recovered from the columns in 30 μ l TE-buffer (10 mM Tris.HCl, 1 mM EDTA (sodium salt), pH 8.0).

Sequencing reactions were done on both strands
10 using the ABI prism BigDye Terminator Cycle sequencing kit and were run on an Applied Biosystems 377XL sequencer (Perkin Elmer, ABI Division, Foster City, CA, USA). The SequencherTM software was used for sequence assembly and manual editing (GeneCodes,
15 AnnArbor, MI, USA).

Cloning of a novel GDNF homologue.

A DNA region spanning nucleotides 67411 to 68343
20 of EMBL accession no. AC005038 of which the translated protein sequence was homologous to mature NTN and PSP was used to design oligonucleotide primers for PCR amplification. The different primers used are shown in Table 1.

25

Table 1: Primers used for the PCR amplification of fragments of AC005038.

	Primer name	Primer sequence
5	PNHsp1	5' - CGGTGCACTCAGGTGATTCCTCC - 3'
	PNHsp2	5' - GGCAGCAAACCCATTATACTGGAACC - 3'
	PNHsp3	5' - CGCTGGTGCAGTGGAAGAGCC - 3'
	PNHsp4	5' - CTGCACCCCCATCTGCTCTTCC - 3'
	PNHap1	5' - GCAGGAAGAGCCACCGGTAAGG - 3'
10	PNHap2	5' - CCAGTCTGCAAAGCCCTGGAGC - 3'

Primers PNHsp3 and PNHap1 were used to amplify a fragment of 502 bp on cDNA derived from different human tissues (fetal brain, whole fetus, prostate or lung Marathon-Ready™ cDNA (Clontech Laboratories) , frontal cortex, hippocampus and cerebellum cDNA) and on human genomic DNA. Based on the genomic sequence from the EMBL/GenBank database (acc. no. AC005038), the fragment to be amplified was predicted to have a G+C content of 76%. Therefore, amplifications were done using the Advantage-GC cDNA PCR kit (Clontech Laboratories, Palo Alto, CA, USA) optimized for the amplification of GC-rich DNA sequences. PCR reactions were performed in a total volume of 50 µl, containing 1x GC cDNA PCR reaction buffer, 0.2 mM dNTP, 1 M GC-MELT™, 200 nM of primers PNHsp3 and PNHap1, 1 µl of Advantage KlenTaq polymerase mix and 1 to 5 µl of cDNA or 0.5 µg of genomic DNA. Samples were heated to 95°C for 5 min and cycling was done for 45 s at 95°C, 1 min at 58°C and 40 s at 72°C for 35 cycles, with a final step of 7 min at 72°C. Samples were finally treated with 2.5 U of native Taq DNA polymerase to add an A-

overhang. PCR products were analysed on a 1% (w/v) agarose gel in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA (sodium salt), pH 8.3). PCR fragments of the expected size (495 bp) were excised from the gel and
5 purified with the Qiaquick gel extraction kit. The PCR fragments were sequenced to confirm their identity and cloned into the plasmid vector pCR2.1-TOPO using the TOPO TA cloning kit according to manufacturer's instructions. Approximately 20 ng of purified fragment
10 was combined with 1 μ l pCR2.1-TOPO vector in a total volume of 5 μ l. Reactions were incubated at room temperature (20°C) for 5 min. 2 μ l of the reaction was transformed into TOP10F' competent cells (Invitrogen BV) using heat-shock transformation and plated on 2x
15 YT/ampicillin plates supplemented with 10 mM IPTG and 2% (w/v) X-gal for blue-white screening. White colonies after overnight growth were picked from the plates, grown in 5 ml of LB medium supplemented with 100 mg/l ampicillin and plasmid DNA prepared using the
20 Qiaprep Spin Miniprep kit. The presence of an insert of the expected size was confirmed by restriction digestion with *EcoRI*. The plasmid insert of several positive clones was sequenced and the obtained sequences compared using the ClustalW alignment
25 program.

To obtain additional coding sequence for the novel GDNF homologue, a fragment with an expected size of 931 bp based on the EMBL/GenBank sequence (acc. no. AC005038) was amplified by PCR using primers PNHspl
30 and PNHapl. PCR reactions were performed in a total volume of 50 μ l, containing 1x GC cDNA PCR reaction buffer, 0.2 mM dNTP, 1 M GC-MELT™, 200 nM of primers PNHspl and PNHapl, 1 μ l of Advantage KlenTaq

polymerase mix and 1 to 5 μ l of cDNA from cerebellum, frontal cortex or hippocampus or 0.5 μ g of genomic DNA. Samples were heated to 95°C for 5 min and cycling was done for 45 s at 95°C, 1 min at 58°C and 1 min 30 s at 72°C for 35 cycles, with a final step of 7 min at 72°C. PCR products were analysed on a 1% (w/v) agarose gel in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA (sodium salt), pH 8.3). A second round amplification was performed with nested primers (PNHsp2 and PNHap2). 1 μ l of the first round amplification reaction was used in a total volume of 50 μ l, containing 1x GC cDNA PCR reaction buffer, 0.2 mM dNTP, 1 M GC-MELT™, 200 nM of primers PNHsp2 and PNHap2 and 1 μ l of Advantage KlenTaq polymerase mix. Samples were heated to 95°C for 5 min and cycling was done for 45 s at 95°C, 1 min at 58°C and 1 min 30 s at 72°C for 35 cycles, with a final step of 7 min at 72°C. Samples were analysed on a 1% (w/v) agarose gel in 1x TAE buffer. PCR fragments of the expected size (870 bp) were excised from the gel and purified with the Qiaquick gel extraction kit. The PCR fragments were sequenced to confirm their identity, treated with 2.5 U of Taq polymerase and cloned into the plasmid vector pCR2.1-TOPO using the TOPO TA cloning kit according to manufacturer's instructions. Approximately 20 ng of purified fragment was combined with 1 μ l pCR2.1-TOPO vector in a total volume of 5 μ l. Reactions were incubated at room temperature (20°C) for 5 min. 2 μ l of the reactions was transformed into TOP10F' competent cells using heat-shock transformation and plated on 2x YT/ampicillin plates supplemented with 10 mM IPTG and 2% (w/v) X-gal for blue-white screening. White colonies after overnight growth were picked from the

plates, grown in 5 ml of LB medium supplemented with
100 mg/l ampicillin and plasmid DNA prepared using the
Qiaprep Spin Miniprep kit. The presence of an insert
of the expected size was confirmed by restriction
5 digestion with *EcoRI*. The plasmid insert of several
positive clones was sequenced and the sequences
compared using the ClustalW alignment program.

**Analysis of enovin gene expression by
RT-PCR analysis.**

Oligonucleotide primers PNHsp3 and PNHap1 (see
table 1) were used for the specific PCR amplification
of a 502 bp fragment from enovin. PCR amplifications
15 were performed on human multiple tissue cDNA (MTC™)
panels normalised to the mRNA expression levels of six
different housekeeping genes. PCR reactions with
enovin specific primers were performed in a total
volume of 50 µl, containing 5 µl of cDNA, 1x GC cDNA
20 PCR reaction buffer, 0.2 mM dNTP, 1 M GC-MELT TM, 400
nM of primers PNHsp3 and PNHap1 and 1 µl of Advantage
KlenTaq polymerase mix. samples were heated to 95°C
for 30 s and cycling was done for 30 s at 95°C and 30
s at 68°C for 35 cycles. Samples were analysed on a
25 1.2 % (w/v) agarose gel in 1x TAE buffer (40 mM Tris-
acetate, 1 mM EDTA (sodium salt), pH 8.3) and images
of the ethidium bromide stained gels were obtained
using an Eagle Eye II Video system (Stratagene, La
Jolla, CA, USA).

30 Similarity searching of the daily update of the
EMBL/GenBank database with the human neurturin and
persephin protein sequences yielded a genomic DNA

sequence coding for a putative novel protein similar to the neurotrophic factors *GDNF*, *NTN* and *PSP* which has been called enovin (EVN). Additional database homology searching using the genomic DNA sequence surrounding the region coding for enovin yielded several expressed sequence tag (EST) clones derived from different human tissues (prostate epithelium [accession no. AA533512 (ID1322952)], lung carcinoma [accession no. AA931637] and parathyroid tumor [accession no. AA844072]). These clones contain DNA sequence outside of the region of homology with *GDNF*, *PSP* or *NTN*, but confirmed that enovin mRNA is expressed in normal and tumor tissues.

Initial PCR amplification using primers (PNHsp3 and PNHapl) based on the genomic sequence yielded a fragment of \approx 500 bp from fetus, fetal brain, prostate, frontal cortex, hippocampus, cerebellum cDNA and from genomic DNA, but not from lung cDNA. Cloning and sequence analysis of these fragments yielded a DNA sequence of 474 bp that translated into a predicted protein sequence of 139 amino acid residues including seven conserved cysteine residues characteristic of all the members of the transforming growth factor β (TGF- β) family of proteins (Kingsley, 1994) (Figure 1). The sequence also contained a RXXR motif for cleavage of the prodomain (RAAR, amino acid position 23 to 26) (Barr, 1991). A similar cleavage site is present in the *GDNF*, *NTN* and *PSP* protein sequences, at a comparable position in the prodomain sequence. Assuming cleavage of the enovin prodomain occurs at this site *in vivo*, the mature EVN protein sequence contains 113 amino acid residues (residue 27 to 139 in figure 1) and has a calculated molecular mass of 11965

Da and an isoelectric point of 11.8. There is one potential N-glycosylation site present in the mature sequence (NST at amino acid position 121-123).

Moreover, several regions conserved between the mature forms of the known neurotrophic factors GDNF, NTN and PSP were also present in enovin (Figure 2). Table 2 summarizes the results of the comparison of the mature protein sequences of the GDNF family members by the BESTFIT program. Percentage identity and percentage similarity are shown. The GDNF, NTN, PSP and EVN mature sequences used in this comparison started at the first amino acid residue following the RXXR cleavage site.

Table 2: Pairwise comparison of mature human GDNF family members using the BESTFIT program.

Comparison	% identity	% similarity
EVN vs GDNF	38.8	47.2
EVN vs NTN	51	56.1
EVN vs PSP	53.3	57.8
GDNF vs NTN	44.8	57.3
GDNF vs PSP	44.3	50
NTN vs PSP	50	54.4

From these comparisons it is apparent that the mature enovin protein is more closely related to persephin and to neurturin than to GDNF.

Amplification, cloning and sequence analysis of a larger fragment of the enovin DNA sequence from frontal cortex cDNA using primers based on the genomic EMBL/GenBank sequence (acc. no. AC005038) yielded a

sequence of 819 bp (Figure 3). This sequence contains
a putative ATG start codon at nucleotide positions 30-
32 and yields an open reading frame (reading frame A
in figure 3) that extends up to a stop codon at
5 nucleotide positions 285-287. The translated protein
sequence of this region does not show similarity to
any known protein in the databases. Translation of the
cDNA sequence in the second reading frame (reading
frame B in figure 3) yields a predicted protein
10 sequence of 159 amino acid residues. This sequence
contains the RXXR cleavage site (position B43 to B46;
nucleotide position 460-471) and the sequence
corresponding to the mature enovin sequence (position
B47 to B159; nucleotide position 472-810). The open
15 reading frame including the RXXR cleavage site and the
mature enovin coding sequence extends from nucleotide
position 334 (preceded-by an in-frame stop codon) to a
stop codon at position 811-813, but does not contain
an ATG codon for a starting methionine residue. In
20 analogy with persephin (Milbrandt et al., 1998) we
hypothesize that an unspliced intron is present in the
majority of the mRNA transcripts from the EVN gene.
GDNF and NTN also have an intron in their respective
prodomain coding regions (Matsushita et al., 1997,
25 Heuckeroth et al., 1997).

To evaluate the existence of different mRNA
transcripts for Enovin, RT-PCR experiments were
performed using primers situated at the 5' end of the
Enovin coding sequence just 5' of a possible upstream
30 ATG start codon (primer PNHsp5 [5'-GCA AGC TGC CTC AAC
AGG AGG G-3'] and nested primer PNHsp6 [5'-GGT GGG GGA
ACA GCT CAA CAA TGG-3'] and at the 3' end (primer
PNHap1 and nested primer PNHap2 [see Table 1]).

Experiments were performed on human multiple tissue cDNA panels (Clontech MTC panels I and II), on a fetal heart cDNA library (Clontech) and on cDNA derived from human cerebellum, hippocampus or frontal cortex

5 (Masure et al., 1998). Primary PCR reactions were performed with primers PNHsp5 and PNHap1 under GC-rich conditions (Advantage GC-PCR kit, Clontech) for 30 cycles (95°C - 30s, 60°C - 30s, 72°C - 1 min) as described. Nested PCR reactions were performed on 1
10 μ l of the primary PCR product using primers PNHsp6 and PNHap2 under the same conditions for 30 cycles. Resulting PCR products were analysed on a 1.5% agarose gel and ranged in size from \pm 350 bp to \pm 800 bp. Several bands were purified from the gel and the PCR
15 fragments sequenced directly. Some purified PCR products were also cloned in vector pCR2.1-TOPO (TOPO-TA cloning kit, Invitrogen) and then sequenced. Sequence analysis confirmed the existence of different mRNA molecules containing Enovin sequence. The
20 obtained fragment sequences were compared with the genomic Enovin sequence. This allowed us to identify several possible 5' and 3' splice sites in the genomic sequence (Figure 21). All these splice sites corresponded to the consensus sequences for donor and
25 acceptor splice sites (Senapathy, P., Shapiro, M.B. & Harris, N.L. (1990)) splice junctions, branch point sites, and exons: sequence statistics, identification, and applications to genome project. Methods Enzymol. 183,252-278). The different Enovin splice variants
30 identified and their presence in different human tissues are summarized in Figure 22. Only two of the 5 sequenced transcripts yield functional Enovin protein upon translation from the ATG start codon.

These two transcripts code for proteins of 228 or 220 amino acids, respectively with predicted signal peptides of 47 and 39 amino acid residues. The predicted protein sequences of these two variants are shown in Figure 23 (long variant) and Figure 24 (short variant). The long variant can be deduced from the DNA sequence of Figure 21 by splicing out the first intron at locations 5'-1 and 3'-1 and the second intron at 5'-2 and 3'-3. Upon translation of the open reading frame, the predicted protein sequence of Figure 23 is obtained. The shorter variant can be deduced from the DNA sequence of Figure 21 by splicing out the first intron at locations 5'-1 and 3'-2 and the second intron at 5'-2 and 3'-3. Upon translation of the open reading frame, the predicted protein sequence of Figure 24 is obtained.

The longest transcript seems to be the most abundant in most tissues as judged by the band intensity in Figure 22B. The shorter transcripts result in frame shifts yielding a translated protein missing the mature Enovin amino acid sequence homologous with GDNF, NTN and PSP. The two smallest transcripts even miss part of the mature coding sequence, including two of the seven highly conserved cysteine residues. Figure 22B shows the distribution of the main splice variants in different human tissues. Functional Enovin mRNA is expressed in almost all tissues tested, including brain, heart, kidney, liver, lung, pancreas, skeletal muscle, colon, small intestine, peripheral blood leukocytes, spleen, thymus, prostate, testis, ovary, placenta and fetal heart. In some human tissues (e.g. cerebellum, hippocampus), only non-functional transcripts could be

amplified by PCR. To our knowledge, the occurrence of non-functional mRNA transcripts to such an extent has not been described before. The biological significance of this finding remains to be studied.

5 Although the expression of NTN and PSP in different tissues has not been fully characterized, their expression levels seem much lower and the expression more restricted to certain tissues (Kotzbauer et al., 1996, Milbrandt et al., 1998).

10

Recombinant expression of Enovin in E. coli
Construction of an Enovin expression plasmid

15 A 414 bp PCR fragment was amplified from human genomic DNA with primers PNHsp4 and PNHap2 (Table 1) and cloned in vector pCR2.1-TOPO using TA-cloning (Invitrogen). The sequence of the insert was confirmed by sequence analysis. One clone containing an-insert with the Enovin consensus sequence (clone 36) was used
20 for subsequent construction of an expression plasmid. Two primers were designed containing appropriate restriction sites at their 5' ends. Forward primer PNHexp-sp1 (5'- GCG GAT CCG GCT GGG GGC CCG GGC A -3') contained a BamHI restriction site (underlined) and
25 reverse primer PNHexp-ap1 (5'- GCC TCG AGT CAG CCC AGG CAG CCG CAG G -3') contained a XhoI restriction site (also underlined). Using these primers, the 343 bp fragment coding for mature Enovin (position 81 to 422 in Figure 1) was amplified from clone 36. The PCR
30 reaction was performed in a total volume of 50 μ l, containing 1x GC cDNA PCR reaction buffer, 0.2 mM dNTP, 1 M GC-MELT™, 200 nM of primers PNHexp-sp1 and

PNHexp-ap1, 1 μ l of Advantage KlenTaq polymerase mix
and 10 ng of plasmid DNA from clone 36. Samples were
heated to 94°C for 5 min and cycling was done for 45 s
at 94°C, 1 min at 58°C and 30 s at 72°C for 25 cycles,
5 with a final step of 7 min at 72°C. The resulting 50
 μ l product was purified using the Qiaquick PCR
purification kit (Qiagen) and the DNA eluted in 30 μ l.
25 μ l of this purified product was then digested in a
30 μ l reaction with 10 U of BamHI and 10 U of XhoI in
10 1x buffer B (Boehringer Mannheim) for 1 h at 37°C.
After electrophoresis in a 1% (w/v) agarose gel in 1x
TAE buffer (40 mM Tris-acetate, 1 mM EDTA (sodium
salt), pH 8.3), the expected 353 bp band was excised
from the gel and purified using the Qiaquick gel
15 extraction kit. The resulting fragment was ligated in
the vector pRSET B (Invitrogen) linearised with BamHI
and XhoI. The insert of the resulting plasmid
construct (hEVNmat/pRSETB) was confirmed by complete
sequence analysis. The resulting construct codes for a
20 146 amino acid protein with a predicted molecular mass
of 15704 Da including an NH2-terminal 6x His-tag fused
in frame to the mature Enovin coding sequence. The
NH2-terminal amino acid sequence of the resulting
protein is thus
25 MRGSHHHHHHHGMASMTGGQQMGRDLYDDDDKDPAGGPGS (mature Enovin
sequence in bold, 6x His tag underlined).

Expression of Enovin in BL21(DE3) E. coli cells

30 Recombinant production of Enovin protein was
performed essentially as described for Neurturin by
Creedon et al. (1997), with modifications. For the

production of recombinant Enovin protein, the plasmid hEVNmat/pRSETB was transformed in E. coli strain BL21(DE3) (Novagen) and grown in 2xYT/ampicillin-medium (16 g/l of tryptone, 10 g/l of yeast extract, 5 g/l of NaCl and 100 mg/l of ampicillin) at 30°C (225 rpm) or 37°C (300 rpm) to an OD600 of approximately 0.5 prior to addition of IPTG to a final concentration of 0.2 mM to induce expression. Cell pellets were harvested by centrifugation following a 3 h induction period, washed with phosphate-buffered saline, centrifuged and stored frozen. For purification and refolding, cell pellets were resuspended in sonication buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM 2-mercaptoethanol, protease inhibitors (Complete™ protease inhibitor cocktail tablets (Boehringer Mannheim, 1 tablet per 50 ml buffer) and 1 mg lysozyme per 500 mg cell pellet). Cells were disrupted by sonication and inclusion bodies harvested by centrifugation. Inclusion bodies were dissolved and incubated in buffer A (8 M urea, 20 mM Tris-HCl pH 7.6, 200 mM NaCl, 1 mM 2-mercaptoethanol) for 30 min at 37°C prior to adding to Ni-NTA resin (nickel nitrilotriacetic acid, Qiagen). After 40 min shaking at 37°C, samples were washed once with buffer A and loaded onto a 5 ml Ni-NTA column. The column was washed successively with 10 column volumes of buffer A, 10 column volumes of buffer A at pH 7.2 and 10 column volumes of buffer A at pH 7.2 + 10 mM imidazole. The Enovin was eluted from the column in 4 column volumes of buffer A at pH 7.2 + 200 mM imidazole.

Enovin refolding was performed by stepwise overnight dialysis at 4°C in renaturation buffer (0.1M

sodium phosphate, 0.15M NaCl, 3 μ M cysteine, 0.02% Tween-20, 10% glycerol, 0.01M Tris-HCl, pH 8.3) containing decreasing amounts of urea at each step (6M to 4M to 3M to 2M to 1M to 0.5M to 0M urea). The
5 purified protein was aliquotted, stored at -20°C and further used for functional assays.

Chromosomal localization of the Enovin gene.

10 A 3.3 kb fragment of the Enovin gene was amplified from cerebellum cDNA using primers EVN(7)-spl (5'- TTC GCG TGT CTA CAA ACT CAA CTC CC
-3') and PNHapl (5'- GCA GGA AGA GCC ACC GGT AAG G
-3') designed on the sequence of EMBL accession number
15 AC005038. The PCR reaction was performed in a total volume of 50 μ l, containing 1x Expand Long Template PCR reaction buffer (Boehringer Mannheim), 0.5 mM dNTP, 1 M GC-MELT((Clontech Laboratories), 400 nM of primers EVN(7)-spl and PNHapl and 1 μ l of cerebellum
20 cDNA. After an initial 2 min at 94°C, 0.75 μ l of Expand Long Template polymerase (Boehringer Mannheim) was added and cycling was done for 10 s at 94°C, 30 s at 58°C and 3 min at 68°C for 10 cycles. Then, 20 additional cycles were performed increasing the
25 extension time at 68°C with 20 s every cycle. A final 7 min at 68°C were also included. The resulting 3.3 kb fragment was purified after electrophoresis in a 0.8% agarose/TAE gel and cloned in vector pCR2.1-TOPO using TA-cloning (Invitrogen). Complete sequence analysis of
30 the 3.3 kb insert of one clone confirmed that the obtained cDNA sequence corresponded to the genomic sequence in the EMBL database (accession number AC005038). No introns were spliced out in the cDNA

obtained from cerebellum cDNA.

Chromosomal mapping studies were carried out using fluorescent in situ hybridization (FISH) analysis essentially as described (Heng et al., 1992, Heng & Tsui, 1993). Human lymphocytes were cultured at 37°C for 68-72 h before treatment with 0.18 mg/ml 5-bromo-2'-deoxyuridine (BrdU) to synchronize the cell cycles in the cell population. The synchronized cells were washed and recultured at 37°C for 6 h. Cells were harvested and slides were prepared using standard procedures including hypotonic treatment, fixation and air-drying. The 3.3 kb probe for Enovin was biotinylated and used for FISH detection. Slides were baked at 55°C for 1 h, treated with RNase and denatured in 70% formamide in 2x NaCl/Cit (20x NaCl/Cit being 3 M NaCl, 0.3 M disodium citrate, pH 7.0) for 2 min at 70°C followed by dehydration with ethanol. The probe was denatured prior to loading on the denatured chromosomal slides. After overnight hybridization, slides were washed and FISH signals and the 4',6-diamidino-2-phenylindole banding pattern were recorded separately on photographic film, and the assignment of the FISH mapping data with chromosomal bands was achieved by superimposition of FISH signals with 4',6-diamidino-2-phenylindole banded chromosomes (Heng & Tsui, 1993). Under the conditions used, the hybridization efficiency was approximately 72% for this probe (among 100 checked mitotic figures, 72 of them showed signals on one pair of the chromosomes). Since the 4',6-diamidino-2-phenylindole banding was used to identify the specific chromosome, the assignment between the signal from the probe and the short arm of chromosome 1 was obtained. The detailed

position was further determined based upon the summary from 10 photographs (Figure 4A). There was no additional locus picked by FISH detection under the conditions used, therefore, we conclude that Enovin is located at human chromosome 1, region p31.3-p32. An example of the mapping results is presented in Figure 4B.

From the gene map data at the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/genemap>), it can be deduced that the genomic clone containing the Enovin sequence (EMBL accession number AC005038) is located on chromosome 1, between markers D1S2843 and D1S417. This corresponds to chromosome 1, region p31.1 to p32.3, confirming the data obtained by FISH analysis.

Tissue distribution of Enovin as determined by Northern blot and dot blot analysis.

Northern blots containing 2 μ g of poly(A)-rich RNA derived from different human tissues (Clontech Laboratories, Palo Alto, CA, USA; MTNTM blot, MTNTM blot II and Fetal MTNTM blot II) were hybridised according to the manufacturer's instructions with a (α -³²P-dCTP random-priming labelled (HighPrime kit, Boehringer Mannheim) 897 bp Enovin fragment. This fragment was obtained by PCR amplification with primers PNHspl and PNHapl on frontal cortex cDNA and subsequent cloning in vector pCR2.1-TOPO. The fragment contains 897 bp of Enovin sequence up to the stop codon and includes the complete coding sequence for the mature Enovin protein.

Enovin mRNA was detected as a main transcript of approximately 4.5 kb (Fig. 5A-C). Enovin mRNA was expressed in a range of tissues, most prominently in heart, skeletal muscle, pancreas and prostate. Some smaller-sized transcripts are present in e.g. placenta (4 kb, 2.4 kb and 1.6 kb) and prostate (4 kb and 1.6 kb). In fetal tissue, a prominent 2.4 kb transcript is present in liver and to a lesser extent lung. Other transcripts are also present in fetal kidney, liver, lung and brain.

In addition an RNA master blot (Clontech Laboratories) containing poly(A) rich RNA from different human tissues and developmental stages was also hybridized with the 897 bp Enovin probe. The poly(A) rich RNA samples used for making this blot have been normalized to the mRNA expression levels of eight different housekeeping genes by the manufacturer. Enovin mRNA was expressed ubiquitously, but highest mRNA levels were apparent in prostate, pituitary gland, trachea, placenta, fetal lung, pancreas and kidney (Figure 5D+E).

Construction of GFR α -IgG-Fc fusions vectors

cDNA regions of GFR α -1, GFR α -2 and GFR α -3 (coding for amino acids 27 to 427, 20 to 431 and 28 to 371, respectively) excluding the sequences coding for the signal peptide and for the COOH-terminal hydrophobic region involved in GPI-anchoring were cloned in-frame in the expression vector Signal pIg plus (R&D Systems Europe Ltd). The resulting proteins expressed from these constructs contain a 17 amino acid NH₂-terminal CD33 signal peptide, the GFR α protein region and a 243

amino acid COOH-terminal human IgG₁-Fc fusion domain.
CHO cells were transfected with GFR α fusion constructs
and stably transfected cells were selected using 500
 μ g G418. Permanent clones were selected using anti Fc
5 antibody. For purification of GFR α fusion proteins,
cells were grown in serum-free medium and medium was
collected after every 3 days. Medium was centrifuged
and applied to a protein A column (Protein A
Sephacrose, Pharmacia Biotech). Bound protein was
10 eluted with 0.1 M Na citrate, pH 3.0 and collected
into 1 M Tris buffer, pH 8.4. Protein concentration
was estimated by absorbance at 280 nm using an
extinction coefficient of 1.5. These purified soluble
GFR α -1 to -3 Fc fusion proteins were used for
15 subsequent binding studies.

Surface plasmon resonance analysis

Surface plasmon resonance (SPR) experiments were
20 performed at 25°C using a BIAcore 3000 instrument.
Analyses were performed with enovin and NGF as
immobilised ligands. The carboxylated matrix of a F1
sensor chip was first activated with a 1:1 mixture of
400 mM N-ethyl-N-(dimethylaminopropyl)-carbodiimide
25 and 100 mM N-hydroxy-succinimide for 10 min. Then,
recombinant enovin and NGF were applied onto the
activated surface in 10 mM acetate buffer, pH 4.5 at a
flow rate of 5 μ l/min. Unoccupied reactive groups were
inactivated with 1 M ethanolamine hydrochloride. For
30 binding experiments, soluble GFR α 1-3-Fc were
superfused at concentrations of 10-100 nM in HEPES
buffered saline (150 mM NaCl, 3.5 mM EDTA, 0.05 %
P-20, 10 mM HEPES, pH 7.4) at a flow rate of 10

5 $\mu\text{l}/\text{min}$. The association was monitored for 3 min and dissociation for 1 min, followed by regeneration with 5 mM NaOH. Dissociation was initiated by superfusion with HEPES buffered saline. A BIAcore evaluation software, 3.0 was used to calculate the association rate (k_a), dissociation rate (k_d) and the equilibrium dissociation constants (K_D , calculated as k_d/k_a).

Results

10

SPR was used to measure binding of soluble GFR α 1-3 to immobilised enovin. Specific binding to enovin could be detected with the soluble GFR α 3 only. GFR α 1 and GFR α 2 did not bind to the immobilised enovin. The observed binding of GFR α 3 was specific as there was no binding to NGF. In the separate control experiment specific binding of TrkA-Fc (NGF receptor) to the immobilised NGF was detected without binding to immobilised enovin.

20

From the binding curves obtained using three different concentrations of GFR α , the following constants in Table 3 were derived. These results demonstrate that GFR α 3 binds specifically to enovin.

25

Table 3

	K_a (1/Ms)	K_d (1/s)	K_D (M)
GFR α 3	$1.65 \cdot 10^5$	$5.08 \cdot 10^{-4}$	$3.1 \cdot 10^{-9}$

30

Since GDNF, NTN and PSP all promote the maintenance and survival of different types of neuronal cells, it is anticipated that enovin has similar biological effects on nerve cells and,

possibly, on other cell types too. Therefore, it is envisaged that the enovin protein may be useful in the treatment of neural disorders in general, including Parkinson's disease, Alzheimer's disease, peripheral neuropathy, amyotrophic lateral sclerosis (ALS), Huntington's disease, acute brain injury, nervous system tumors, multiple sclerosis, peripheral nerve trauma or injury and exposure to neurotoxins.

Enovin could also be useful in various aspects of neuroprotection. Considering its effect on survival of different neuronal cell populations and on the observed neurite extensions in our model of SHSY5Y cells, we propose that this compound could have neuroprotective and neuroregenerative applications.

This is based upon the following observations. Taxol induces neuronal apoptosis in NGF-differentiated PC12 rat pheochromocytoma cells (Nuydens et al, submitted). Therefore, taxol induced cytotoxicity has features of neuronal apoptosis, as monitored by DNA fragmentation, Annexin V labelling and bcl-2 protection. As an extension, therefore, it can be deduced that taxol induces apoptosis in differentiated SH-SY5Y cells. Enovin is now shown to be able to reduce this cell death and therefore may reverse neuronal apoptosis in general.

The compound may therefore be helpful in the following neurodegenerative conditions in which apoptosis has been observed, stroke (Hakim 1998), Parkinson's disease (Marsden et al 1998), Alzheimer's disease (Nagy et al 1998), Huntington's disease (Wellington et al. 1997), Neurotrauma (Smirnova et al. 1998), Peripheral neuropathies, (Srinivisan et al. 1998).

As an example for the last clinical indication, we have shown that this neurotrophic factor actually protects differentiated SH-SY5Y human neuroblastoma cells against taxol-induced cell toxicity.

5

Methodology of Viability measurements

Cell viability was determined by adding 100 μ l of a 1 mg/ml 2,3-bis [2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide (XTT, Sigma) solution in DMEM (37°C) supplemented with 0.02 mM phenazine methosulfate (PMS, Sigma) to each well. The plates were then incubated at 37°C for 2.5 hours. The optical densities were read (Molecular devices) at 450 nm, using 650 nm as a reference. The XTT assay is based on the conversion of the tetrazolium salt XTT into a red colored formazan product. This reaction is performed by mitochondrial enzymes.

20

Methodology of neuronal differentiation

1. Differentiation in human neuroblastoma SHSY5Y cells

SHSY5Y cells are differentiated for 5 days with 25 nM staurosporine. Effect of Enovin is measured 72 hrs after start of the experiment. (reference Jalava et al. "Protein Kinase inhibitor staurosporine induces a mature neuronal phenotype in SH-SY5Y human neuroblastoma cells through an a,b,z PKC independent pathway" Journ cell Physiol 155, 301-312 (1993)).

30

2. Measurement of neurite extension.

Morphological changes of neurones were

automatically quantified as follows. Briefly, at the appropriate times, glutaraldehyde was added directly to the medium and left for 30 minutes at room temperature. This ensured that the morphology of the cells at that time point reflected the real situation. The cells were observed through transmitted light mode in an Axiovert microscope (Zeiss Oberkochen, Germany), equipped with a Marzhauser scanning stage driven by an Indy workstation (Silicon Graphics, Mountain View, USA). Images were captured using a MX5 video camera (HCS). About 3000 cells were evaluated in 64 aligned images, forming a 8x8 square matrix of images. The exact alignment of the images ensured that neurites could be followed from one image field into the next. Automatic detection of the neurite extensions, labeled by polyclonal tau antibody was performed using an unbiased detector of curvilinear structures (Steger 1998). The analysis software automatically calculated total cell body area, number of cell bodies and total neurite length.

To investigate the effect of enovin on different cell types, two assays were performed, a DNA synthesis assay and a chemotaxis assay.

DNA synthesis assay

Cells including human dermal fibroblasts (39SK), human umbilical vein endothelial cells (HUVEC), human smooth muscle cells (HSMC), human chondrocytes, and rat osteoblasts were maintained in DMEM containing 10% FBS (39-SK, HSMC, rat osteoblasts) or defined media (chondrocytes and HUVEC) at 37 °C with 5% CO₂ and 95% air. For the DNA synthesis assay, cells were seeded

in a 96-well tissue culture plate at a density of
5,000 cells/well in DMEM containing 10% FBS and
incubated for 24 h. The culture medium then was
replaced with DMEM containing various concentrations
5 of Enovin and 0.1% BSA (for 39-SK, osteoblasts, HSMC,
chondrocytes) or DMEM containing various
concentrations of Enovin and 0.5% FBS (for HUVEC) and
cells were incubated for 24 h. Subsequently, the
culture medium was replaced with 100 μ l of DMEM
10 containing 5% FBS and 0.1 μ Ci of [3 H]-thymidine.
Following 2 h of pulse labelling, cells were fixed
with methanol/acetic acid (3:1, vol/vol) for 1 h at
room temperature. The fixed cells were washed twice
with 80% methanol. The cells were solubilized in
15 0.05% trypsin (100 μ l/well) for 30 min and then in
0.5% SDS (100 μ l/well) for an additional 30 min.
Aliquotes of cell lysates (180 μ l) were combined with
2 ml of scintillation cocktail and the radioactivity
of cell lysates was measured using a liquid
20 scintillation counter (Wallac 1409).

Chemotaxis Assay

Cells were maintained as described in "DNA
25 Synthesis Assay". The chemotactic activity of Enovin
was analyzed using a 12-well modified Boyden Chamber
(McQuillan, D.J., Handley, C.J., Campbell, M.A.,
Bolis, S., Milway, V.E., Herington, A.C., (1986),
"Stimulation of Proteoglycan biosynthesis by serum and
30 insulin-like growth factor-I in cultured bovine
articular cartilage", Biochem. J. 240:423-430). Cells
were trypsinized using 0.05% trypsin and 0.5 mM EDTA

and resuspended in DMEM. To the bottom wells of a Boyden chamber, aliquots of 150 μ l of media containing various concentrations of Enovin were added. A polycarbonate membrane (8 μ m) coated with 0.1 mg/ml of type I collagen was placed on the top of the bottom wells, followed by assembling the top wells. To the top wells, aliquots of 100 μ l of cells (70,000 cells/ml) were added. Following a 6-h incubation period, the apparatus was disassembled. Cells remaining on the top of the membrane were removed. The membrane was fixed with 10% formaldehyde for 15 min, followed by staining with Gill's strength hemotoxylin. Cells were counted under microscopy (250 x magnification), and the average of cell counts from five areas of each well was used. Each experiment was repeated at least four times. The results were expressed as the fold of control (DMEM containing 0.1% BSA).

As illustrated by the results in Figure 8 to 18, enovin has no effect on proliferation in each of the cell types used, or on the migration of HUVEC cells (Figure 14) as described above. There was an effect of enovin on SH-SY-5Y neuroblastoma cells. This demonstrated enovins selective effect on neuronal cells.

Both GDNF and NTN have been shown to signal via a signalling complex composed of a ligand-binding subunit, either GFR α -1 or GFR α -2, and a signalling subunit, the cRET protein tyrosine kinase. Enovin is expected to exert its biological effects via a similar signalling complex composed of a GFR α binding partner (either GFR α -1, GFR α -2, the recently characterised orphan receptor GFR α -3 or other as yet uncharacterized

members of the GFR α family) in combination with cRET or another signalling partner. Indeed, our binding data show that enovin can bind specifically to GFR α -3.

In humans, germ line mutations in GDNF or cRET
5 can lead to several disease phenotypes including multiple endocrine neoplasia and Familial Hirschsprung disease (HSCR) (Romeo et al., 1994, Ederly et al., 1994, Angrist et al., 1996). Both diseases are associated with gut dismotility, with Hirschsprung
10 disease being the most common cause of congenital bowel obstruction in infants. Interestingly, GDNF and cRET knockout mice exhibit remarkably similar pathologies with renal agenesis and intestinal aganglionosis (Sanchez et al., 1996; Moore et al.,
15 1996; Pichel et al., 1996). Enovin could be involved in similar disorders of the gut or the kidneys or, since it is ubiquitously expressed, could be important in the development of other peripheral organs in the body.

20 The interaction of ligands with their receptors is generally achieved by the interaction of specific bonds from particular residues in both proteins. Fragments of a protein can serve as agonists activating the receptor to elicit its growth promoting
25 and survival sustaining effects on cells. Parts of enovin or synthetic peptides based on the enovin protein sequence can therefore be useful as agonists or antagonists to regulate its receptor GFR α 3. Using peptide synthesis or recombinant techniques, hybrid
30 growth factors composed of parts of GDNF, NTN or PSP or any other neurotrophic or growth factor with parts of enovin can be produced to yield a novel synthetic growth factor with new properties.

Two pilot trials were conducted to test whether enovin is able to change the taxol-induced sensory deficits in rats after subplantar injections in rats. In a first experiment, it was tested whether a single treatment with enovin could reverse the taxol-induced sensory deficit, whereas in a second trial it was tested whether enovin could prevent the development of the taxol-induced deficits.

Reversal over time of taxol-induced sensory dysfunction.

Procedure

Male Sprague-Dawley rats, weighing 300 - 340 gram, were used. The animals were housed individually with food and water ad lib. Before the start of the experiment, the animals were placed in standard observation cages and after a habituation period of 15 min, the pin prick reflex was evaluated. To do so, the plantar surface of the right paw of the animal was stimulated with a needle and the reactivity to this pin-prick was scored as either present (score = 1) or absent (score = 0). Within one session, the procedure was repeated three times with a time interval of 1 min between 2 consecutive stimulus presentations; as such the pin prick test consisted of 3 measures of reactivity to a pin prick. Only rats having normal reactions on the 3 pin pricks were included in the experiment.

On the 3 consecutive days in the morning, the animals received daily a subplantar injection of 50 μ l of taxol (3 mg/ml paclitaxel dissolved in cremophor

and dehydrated alcohol plus water) in the right hind paw. During the next morning, the pin prick reflex was re-evaluated and animals not showing any reactivity to the 3 stimulus presentations were selected. These

5 animals were randomly divided in subgroups ($n = 10$ / group) receiving a subplantar injection in the right hind paw of $75 \mu\text{l}$ of either vehicle, saline or 23 or 130 $\mu\text{g/ml}$ enovin. Because no differences were observed between the results of the vehicle and saline treated

10 animals, both groups were joined (control group). At days 1, 4, 5 and 7 after the last treatment, the pin prick test was performed both in the morning (between 8 and 9 a m) and the evening (between 3.30 and 4.30 p m). On day 8, a last pin prick test was taken during

15 the morning. For each animal, the cumulative score of reactivity to the pin prick was measured over time. Because in total 9 pin prick tests (each consisting of 3 pin prick presentations) were performed after the last drug treatment, the maximal score to be reached

20 over the total time period of the experiment is 27.

Results

Repeated subplantar injections of taxol over 3 consecutive days results in an acute inflammatory

25 reaction with a lack of responding to a pin prick stimulation in the majority of animals. A subplantar injection of saline or vehicle did not affect the taxol-induced deficit. At the first measurement, only 4 out of 20 controls showed at least 1 reaction to the

30 three pin pricks and the mean (\pm SEM) pin prick score of the controls at the first measurement was $0.25 (\pm 0.12)$; this in contrast to the starting of the experiment where the mean score was $3.0 (\pm 0.0)$

because all animals responded to the pin prick. Even after 8 days of measurement, the reactivity in the controls was still impaired with 11 out of 20 rats responding at least once and with a mean pin prick score of 0.75 (\pm 0.18). Within this control group, none of the rats displayed a normal reactivity to all 3 stimuli. The cumulative pin prick score of the controls over time is presented in Figure 19. Because the animals were tested 9 times over an 8 days period, the maximal score to be reached with 3 pin pricks at each test is 27. As seen on the graph, a subplantar injection of saline or vehicle was unable to reverse the taxol-induced deficit over the time period tested. The mean total cumulative score of the controls at the end of the experiment was 5.10 (\pm 0.87); being 18.9 % of the maximal score to be reached.

A single subplantar injection of 75 μ l of 23 μ g/ml enovin, resulted after the first measurement in 4 out of 10 rats responding at least once, with a mean pin prick score of 0.70 (\pm 0.33). At day 8, all 10 animals responded at least once to the pin prick, and a normal reactivity was present in 5 out of 10 rats. The average pin prick score of this group at day 8 was 2.20 (\pm 0.29). As compared to the controls, the average cumulative score at the end of the 8 days of measurement was significantly increased (Mann - Whitney U-test, two-tailed, $p < 0.01$), reaching a mean pin prick score of 14.50 (\pm 1.96) (Figure 19). This is 53.7 % of the maximal score.

Also with an subplantar injection of 130 μ g/ml enovin there was improved efficacy against the controls. At the first measurement after 130 μ g/ml enovin, 6 out of 10 rats responded at least once with a

mean pin prick score of 1.10 (\pm 0.35). At day 8, all 10 animals responded to at least one pin prick with a mean score of 2.60 (\pm 0.22). A normal reactivity to the 3 pin pricks was present in 8 out of 10 rats. The average cumulative total pin prick score at the end of the experiment in this group was 17.20 (\pm 1.94). This is 63.7 % of the total possible score and significantly improved as compared to the control group ($p < 0.01$).

Prevention over time of taxol-induced sensory dysfunction.

Procedure

Male Sprague-Dawley rats, weighing 300 - 340 gram, were used. The animals were housed individually with food and water ad lib. Before the start of the experiment, the animals were placed in standard observation cages and after a habituation period of 15 min, the pin prick reflex was evaluated. To do so, the plantar surface of the right paw of the animal was stimulated with a needle and the reactivity to this pin-prick was scored as either present (score = 1) or absent (score = 0). Within one session, the procedure was repeated three times with a time interval of 1 min between 2 consecutive stimulus presentations; as such the pin prick test consisted of 3 measures of reactivity to a pin prick. Only rats having normal reactions on the 3 pin pricks were included in the experiment (pin prick score = 3). After this control measurement, the animals were randomly divided in subgroups ($n = 10$ / group) receiving an subplantar

injection in the right hind paw of 75 μ l of either vehicle, saline or 23 or 130 μ g/ml enovin. Because no differences were observed between the results of the vehicle and saline treated animals, both groups were joined (control group). During the 3 consecutive days, the animals received daily a subplantar injection of 50 μ l of taxol (3 mg/ml paclitaxel dissolved in cremophor and dehydrated alcohol plus water) in the right hind paw. At days 1, 4, 5 and 7 after taxol, the pin prick test was performed both in the morning (between 8 and 9 a m) and the evening (between 3.30 and 4.30 p m). On day 8, a last pin prick test was done during the morning. For each animal, the cumulative score of reactivity to the pin prick was measured over time. Because in total 9 pin prick tests (each consisting of 3 pin prick presentations) were performed after the taxol treatment, the maximal cumulative score to be reached over the total time period of the experiment is 27.

Results

A subplantar injection of saline or vehicle before taxol did not prevent the taxol-induced deficit in the pin prick test. At the first testing after taxol, 8 out of 20 rats responded at least once to the pin prick, with a mean pin prick score of 0.60 (\pm 0.18). At day 8, the taxol-induced deficit was still present, with only 8 out of 20 animals responding and having a mean score of 0.8 (\pm 0.25). Within two animals, a normalised pin prick reflex was present. Over time, the cumulative pin prick score was also reduced, resulting in a mean value of 6.55 (\pm 1.08), which is 24.3 % of the maximal score (Figure 20).

Pretreatment with 23 $\mu\text{g/ml}$ enovin reduced the taxol-induced deficits on the pin prick. At day 1, 8 out of 10 animals responded at least once, and the average pin prick score was $1.70 (\pm 0.40)$. At day 8, all animals were responding with a mean score of $2.50 (\pm 0.27)$. Here 7 animals revealed a normal reactivity on all pin prick exposures. With regard to the cumulative responding over time (Figure 20), the mean total score was significantly improved ($p < 0.01$) over the control level to $18.40 (\pm 1.73)$; this is 68.1 % of the maximal value.

Comparable results were obtained after a pretreatment with 130 $\mu\text{g/ml}$ enovin. Here, 6 out of 10 animals responded during the first testing with a mean pin prick score of $1.70 (+ 0.31)$. At day 8, all animals were reacting at least once to a pin prick stimulation with a mean score of $2.40 (+ 0.22)$ and all 3 reactions were normal in half of the animals. With regard to the cumulative score, the mean score obtained at day 8 is $17.70 (\pm 1.92)$, representing 65.5 % of the total score.

The present series of experiments indicate that a single subplantar injection of enovin is able to reduce the taxol-induced sensory deficits as measured by a pin prick test. Activity is seen when the drug was applied both before and after taxol.

Enovin is a possible candidate for pain syndromes with mainly a peripheral and central neurogenic component, rheumatic/inflammatory diseases as well as conductance disturbances, and can play a modulatory role in sensory processes after transdermal, topical, local, central (such as epidural, intrathecal, and the like) and systemic application.

Further it is worthwhile to use enovin as a diagnostic tool to screen for physiopathological changes in the area's mentioned above.

5 **Comparison of Enovin mRNA expression in normal
versus diseased tissues**

667120" STEZEE60

10 The expression of Enovin mRNA was quantitatively
analysed using the ABI Prism 7700 Sequence Detection
System (TaqMan; Perkin Elmer) using proprietary
technology developed and carried out at Pharmagene
Laboratories Ltd, Royston, United Kingdom. The system
uses a fluorogenic probe to generate sequence specific
fluorescent signals during PCR. The probe is an
15 oligonucleotide with fluorescent reporter and quencher
dyes attached, it is positioned between the forward
and reverse PCR primers. While intact, the intensity
of reporter fluorescence is suppressed by the-
quencher. Should the probe form part of a replication
20 complex, the fluorescent reporter is cleaved from the
quencher by a 5' to 3' exonuclease activity inherent
in Taq polymerase. The increase in fluorescent
reporter signal within a reaction is a direct measure
of the accumulation of PCR product. The starting copy
25 number of an mRNA target sequence (Cn) is established
by determining the fractional PCR cycle number (Ct) at
which a PCR product is first detected - the point at
which the fluorescence signal passes above a threshold
baseline. Quantification of the amount of target mRNA
30 in each sample is established through comparison of
experimental Ct values with a standard curve.

RNA preparation and quality control

Total RNA was isolated from whole and sub-
dissected tissue, using Tri-Zol reagent (Life
5 Technologies, Gaithersburg, MD, USA) according to the
suppliers' protocol. Quality control procedures for
all RNA samples included an assessment of integrity
(intact 18S and 28S ribosomal RNA) and determination
of the presence of high abundance (actin) and low
10 abundance (transferrin receptor) transcripts.

Primer/probe design

A pair of primers and a TaqMan probe were
designed to amplify a specific sequence from Enovin
15

Primer 1: 5' ACGGTCTCTCCAGGTGCTGT 3'

Primer 3: 5' TGCTGCCGACCCACG 3'

Probe 5: 5' CTACGAAGCGGTCTCCTTCATGGACG 3'

20 In addition a pair of primers and a TaqMan probe
were designed which span an intron and amplify a
portion of the human GAPDH gene

Primer 2:

25 5' CAGAGTTAAAAGCAGCCCTGGT 3'

Primer 4:

5' GAAGGTGAAGGTCGGAGTCAAC 3'

Probe 6:

30 5' TTTGGTCCGTATTGGGCGCCT 3'

Probe 5 is labelled with the fluor FAM while
probe 6 is labelled with the fluor VIC.

DNase treatment of total RNA

For each tissue tested 2.2 μ g of total RNA was digested with 2 units of RNase free DNase (Gibco BRL) for 15 minutes at room temperature in a 20 μ l volume of 1x DNase buffer (Gibco BRL). The reaction was stopped by addition of 2 μ l of 25mM EDTA solution. The samples were then incubated at 65°C for 10 minutes to inactivate the enzyme.

First strand cDNA synthesis

For each tissue tested 100ng of total RNA was used as template for first strand cDNA synthesis. The RNA in a volume of 4ml and in the presence of 50nM primers 1 and 2 , 1xPCR buffer II (Perkin Elmer) and 5mM MgCl₂ was heated to 72°C for 5 minutes and cooled slowly to 55°C. After addition of all other reagents, the 6ml reaction was incubated at 48°C for 30 minutes followed by an enzyme inactivation step of 90°C for 5 minutes. The final reaction conditions were as follows: 1xPCR buffer II, 5mM MgCl₂, 1mM dATP, dTTP, dGTP, dCTP, 12.5 units MuLV reverse transcriptase (Gibco BRL).

PCR amplification of first strand cDNA products

The cDNA derived from 100ng total RNA for each sample was subjected to PCR amplification in a single reaction to identify both target and GAPDH transcripts. The final primer/probe concentrations for target were 300nM primer 1, 300nM primer 3 and 200nM probe 5, those for GAPDH were 20nM primer 2, 20nM primer 4 and 100nM probe 6. The final concentration of other reagents in the reaction were 4.5% glycerol, 1 x TaqMan buffer A (Perkin Elmer), 6.25mM MgCl₂, 430M

dATP, dUTP, dGTP, dCTP, 2.5 units AmpliTaq Gold. The PCR amplification was carried out in the ABI 7700 sequence detection system, an initial enzyme activation step of 94°C for 12 min was followed by 45 cycles of 94°C 15 secs, 60°C 1 min (minimum ramp time).

Diseases and tissues tested

Enovin mRNA expression was compared in tissues derived from disease patients and normal control individuals (Figures 25 and 26). The table below shows the diseases and corresponding tissues that have been investigated. For each condition, three diseased and three control samples were analysed.

Patholog	Tissue 1	Tissue 2	Tissue 3
Alzheimer's disease	temporal cortex	hippocampus	occipital cortex
Multiple sclerosis	spinal cord	periventricular white matter	cerebellum
Parkinson's disease	substantia nigra	putamen	cerebellum
Cancer	Colon adenocarcinoma	breast ductal adenocarcinoma	lung squamous cell carcinoma

Statistical analysis

For each group of 3 tissues, the mean and standard deviation were calculated on the Ct values (which are normally distributed) and were then converted into Cn values according to the formula $Cn = 10^{((Ct-40.007)/-3.623)}$. Analysis of variance (ANOVA) was performed on the Ct values also to compare the mean Enovin mRNA expression levels in normal versus

diseased tissues.

Figures 25 and 26 show the mean Enovin mRNA copy numbers (\pm SD; n=3) in diseased versus control tissues. Statistical analysis showed a significant
5 increase in the Enovin expression level in the periventricular white matter of patients with multiple sclerosis ($p = 0.013$). The internal GAPDH control showed no significant difference ($p = 0.79$). Although the Enovin expression level in the periventricular
10 white matter is quite low in normal tissue (270 copies per 100 ng total RNA on average versus 200000 copies of GAPDH), the level is three times higher (825) in patients with multiple sclerosis.

Only one other diseased tissue showed a
15 significant difference versus normal control: in breast ductal adenocarcinoma, the Enovin mRNA expression level is 6 times higher (6000 versus 1000 ; $p = 0.007$), but the GAPDH control value is also significantly increased (165000 versus 44000 ; $p =$
20 0.03), probably representing a general increase in mRNA levels.

In conclusion, we have found Enovin mRNA levels to be upregulated in the periventricular white matter of patients with multiple sclerosis.
25

Use of phospho-specific antibody cell-based ELISA for screening of enovin mimetic on GFR α 3/cRET receptor complex.

Method can also be used for identification of agonist or antagonist of other neurotrophin receptors, such as GFR α 1, GFR α 2, GFR α 4, TrkA, TrkB and TrkC.

Assay

Using this assay we can identify agonist or antagonist compounds of neurotrophic growth factors by measuring the activation of key signalling kinases activated in the neurotrophic pathway or by measuring the activation of cRET receptor kinase. The activation is measured by detecting the amount of phosphorylated kinase or receptor kinase using phospho-specific antibodies. We will use NIH 3T3 cells expressing transiently or permanently TrkA, TrkB, TrkC, GFR α 1/cRET, GFR α 2/cRET, GFR α 3/cRET or GFR α 4/cRET.

The activation of p42/p44 MAP kinase, PKB kinase, c-jun, CREB, JNK/SAPK kinase and other kinases is detected using commercially available phospho-specific antibodies. In addition, cRET activation can be detected using phospho-specific cRET antibody.

The protocol used was as follows:

-Plate NIH 3T3 cells in 96-wells in 10% calf serum, cells have to be 80% confluent before stimulation.

-Next day, replace medium with serum-free medium and starve cells for 18-24 h.

-After starvation stimulate cells with compounds and neurotrophic factors as positive control (10 ng/ml

for neurotrophic factors)

-Fix cells with 4% formaldehyde in PBS at 4°C for 20 min.

5 min. -Wash cells 3x with 200 μ l PBS/0.1% Triton for 5 min.

-Quench the cells with 100 μ l 0.6% H_2O_2 in PBS/0.1% Triton for 20 min.

-Wash cells 3x with 200 μ l PBS/0.1% Triton for 5 min.

10 -Block the cells with 100 μ l 10% foetal calf serum in PBS/0.1% Triton for 60 min.

-Incubate the cells with phosphospecific antibody in 50 μ l 5% BSA//PBS/0.1% Triton, over night at 4°C. Antibody dilution should be experimentally determined, suggested range 1:100-1:250.

15 -Wash cells 3x with 200 μ l PBS/0.1% Triton for 5 min.

-Incubate with secondary antibody HRP linked, dilution 1:100 in 50 μ l 5% BSA/PBS/0.1% Triton, for 1 h at room temperature.

20 -Wash cells 3x with 200 μ l PBS/0.1% Triton for 5 min.

-Dissolve 1 tablet of OPD (Sigma) in 25 ml buffer (3.65 g citric acid- H_2O and 5.9 g $Na_2HPO_4 \cdot 2H_2O$ in 0.5l H_2O , pH 5.6) and add 12.5 μ l H_2O_2 . Add 50 μ l to each well and incubate for 15 min on shaker (200 rpm), covered with aluminium foil.

25 -Stop the reaction with 25 μ l H_2SO_4 .

-Measure $OD_{490-650}$ on the ELISA reader.

30

Mesencephalic dopaminergic neuronal culture

Neuronal culture

5 Neuronal cultures were prepared from the ventral
mesencephalon of foetal rat by enzymatic and
mechanical dispersion. The tissue was collected,
washed in ice-cold Ca^{2+} - and Mg^{2+} -free phosphate
10 buffered saline containing 0.6 % glucose (PBSG) and
incubated for 30 min with PBSG containing 0.1 %
trypsin at 37°C. The cell suspension was plated at a
density of 2.5×10^5 cells/cm² onto 96 well NUNC tissue
culture plates. In advance, culture plates were coated
with poly-L-ornithine and CDM containing 10 % foetal
15 calf serum. The cultures were maintained in chemically
defined medium (CDM), composed of a 1:1 mixture of
Dulbecco's Modified Eagles medium and F12 Nutrient
supplemented with glucose (0.6 %), glutamine (2 mM),
sodium bicarbonate (3 mM), HEPES (5 mM), insulin (25
20 µg/ml), human transferrin (100 µg/ml), putrescine (60
µg/ml), sodium selenite (30 nM), streptomycin (100
µg/ml) and penicillin (100 IU/ml).

Treatment with neurotrophic factors

25 Neurotrophins were dissolved in 0.5 % bovine
serum albumin as a stock. Neurotrophins were added 3 h
after initial plating and after 5 days in culture. The
same amount of 0.5 % bovine serum albumin was added to
30 the control wells.

High-affinity dopamine uptake

Dopamine uptake was measured after 10 days. For the uptake, cells were washed twice with pre-warmed PBS supplemented with glucose (5 mM), ascorbic acid (100 mM) and pargyline (100 mM) and pre-incubated for 10 min with the same solution. The pre-incubation solution was replaced with the same solution containing 50 nM [³H]DA and incubation continued for 15 min at 37°C. Uptake was stopped by 3 rapid washes with ice-cold PBS. The accumulated [³H]dopamine was released by incubating with acidified ethanol for 30 min at room temperature. Radioactivity was determined after addition of 4 ml of scintillation liquid (Packard ultima gold MV) using Packard scintillation counter. Non-specific uptake was determined by adding 20 µM cocaine.

Table 4. Effect of enovin on [³H]dopamine uptake

Treatment	Percent control [³ H]dopamine uptake	n
control	100	5
enovin 300 ng/ml	111	4
25 enovin 1000 ng/ml	127	5
enovin 2000 ng/ml	152	5
enovin 4000 ng/ml	161	1
enovin 10000 ng/ml	165	2

30 Cells were grown for 10 days in the presence or absence of enovin. Untreated controls were set as 100

% . Results are obtained in 1-5 independent experiments.

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References

- 5 Altschul, S.F., Gish, W., Miller, W., Myers, E.W. &
Lipman, D.J. (1990) Basic local alignment search tool,
J. Mol. Biol. 215, 403-410.
- 10 Angrist, M., Bolk, S., Halushka, M., Lapchak, P.A. &
Chakravarti, A. (1996) Germline mutations in glial
cell line-derived neurotrophic factor (GDNF) and RET
in a Hirschsprung disease patient. *Nature Genetics*
14, 341-344.
- 15 Baloh, R.H., Tansey, M.G., Golden, J.P., Creedon,
D.J., Heuckeroth, R.O., Keck, C.L., Zimonjic, D.B.,
Popescu, N.C., Johnson, E.M. & Milbrandt, J. (1997)
TrnR2, a novel receptor that mediates neurturin and
GDNF signaling through Ret. *Neuron* 18, 793-802.
- 20 Baloh, R.H., Gorodinsky, A., Golden, J.P., Tansey,
M.G., Keck, C.L., Popescu, N.C., Johnson, E.M. &
Milbrandt, J. (1998) GFR α 3 is an orphan member of
the GDNF/neurturin/persephin receptor family. *Proc.*
Natl. Acad. Sci. USA 95, 5801-5806.
- 25 Barr, P.J. (1991) Mammalian subtilisins: the long-
sought dibasic processing endoproteases. *Cell*. 66, 1-
3.
- 30 Beck, K.D., Valverde, J., Alexi, T., Poulsen, K.,
Moffat, B., Vandlen, RA., Rosenthal, A. & Hefti, F.
(1995) Mesencephalic dopaminergic neurons protected

by GDNF from axotomy-induced degeneration in the adult brain. *Nature* 373,339-341.

- 5 Bilang-Bleuel, A., Revah, F., Colin, P., Locquet, I.,
Robert J.J., Mallet, J. & Horellou, P. (1997)
Intrastriatal injection of an adenoviral vector
expressing glial-cell-line-derived neurotrophic factor
prevents dopaminergic neuron degeneration and
behavioral impairment in a rat model of Parkinson
10 disease. *Proc. Natl. Acad. Sci. USA* 94,8818-8823.
- Buj-Bello, A., Buchman, V.L., Horton, A., Rosenthal,
A. & Davies A.M. (1995) GDNF is an age-specific
survival factor for sensory and autonomic neurons.
15 *Neuron* 15,821-828.
- Buj-Bello, A., Adu, J., Piñón, L.G.P., Horton, A.,
Thompson, J., Rosenthal, A., Chinchetru, M., Buchman,
V.L. & Davies, A.M. (1997) Neurturin responsiveness
20 requires a GPI-linked receptor and the Ret receptor
tyrosine kinase. *Nature* 387,721-724.
- Choi-Lundberg, D.L., Lin, Q., Chang, Y.N., Chiang,
Y.L., Hay, C.M., Mohajeri, H., Davidson, B.L. & Bohn,
25 M.C. (1997) Dopaminergic neurons protected from
degeneration by GDNF gene therapy. *Science*. 275,838-
841.
- Creedon, D.J., Tansey, M.G., Baloh, R.H., Osborne,
30 P.A., Lampe, P.A., Fahrner, T.J., Heuckeroth, R.O.,
Milbrandt, J. & Johnson, E.M. (1997) Neurturin shares
receptors and signal transduction pathways with glial

cell line-derived neurotrophic factor in sympathetic neurons. Proc. Natl. Acad. Sci. USA 94, 7018-7023.

- 5 Durbec, P., Marcos-Gutierrez, C.V., Kilkenny, C.,
Grigoriou, M., Wartiovaara, K., Suvanto, P., Smith,
D., Ponder, B., Costantini, F., Saarma, M., Sariola,
H. & Pachnis, V. (1996) GDNF signalling through the RET
receptor tyrosine kinase. Nature 381, 789-793.
- 10 Edery, P., Lyonnet, S., Mulligan, L.M., Pelet, A.,
Dow, E., Abel L., Holder S., Nihoul-Fekete, C.,
Ponder, B.A. & Munnich, A. (1994) Mutations of the RET
proto-oncogene in Hirschsprung's disease. Nature
367, 378-380.
- 15 Gash, D.M., Zhang, Z., Ovadia, A., Cass, W.A., Yi, A.,
Simmerman, L., Russell, D., Martin, D., Lapchak, P.A.,
Collins, F., Hoffer, B.J. & Gerhardt, G.A. (1996)
Functional recovery in parkinsonian monkeys treated
20 with GDNF. Nature 380, 252-255.
- GFR α Nomenclature Committee (1997) Nomenclature of
GPI-linked receptors for the GDNF ligand family.
Neuron 19, 485.
- 25 Hakim A "Ischemic penumbra: the therapeutic window. "
Neurology. 1998 Sep;51(3 Suppl 3):S44-6.
- 30 Henderson, C.E., Phillips, H.S., Pollock, R.A.,
Davies, A.M., Lemeulle, C., Armanini, M., Simmons, L.,
Moffet, B., Vandlen, R.A., Koliatsos, V.E. &
Rosenthal, A. (1994) GDNF: a potent survival factor

for motoneurons present in peripheral nerve and muscle. *Science* 266,1062-1064.

5 Heng, H.H.Q., Squire, J. & Tsui, L.-C. (1992) High resolution mapping of mammalian genes by in situ hybridization to free chromatin. *Proc. Natl. Acad. Sci. USA* 89, 9509-9513.

10 Heng, H.H.Q. & Tsui, L.-C. (1993) Modes of DAPI banding and simultaneous in situ hybridization. *Chromosoma* 102, 325-332.

15 Heuckeroth, R.O., Kotzbauer, P., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Zimonjic, D.B., Popescu, N.C., Johnson, E.M. & Milbrandt, J. (1997) Neurturin, a novel neurotrophic factor, is localized to mouse chromosome 17 and human chromosome 19p13.3. *Genomics* 44,137-140.

20 Jing, S., Wen, D., Yu, Y., Holst, P.L., Luo, Y., Fang, M., Tamir, R., Antonio, L., Hu, Z., Cupples, R., Louis, J.-C., Hu, S., Altrock, B.W. & Fox, G.M. (1996) GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR- α , a novel receptor for
25 GDNF. *Cell* 85, 1113-1124.

Jing, S., Yu, Y., Fang, M., Hu, Z., Holst, P.L., Boone, T., Delaney, J., Schultz, H., Zhou, R. & Fox, G.M. (1997) GFR α -2 and GFR α -3 are two new receptors
30 for ligands of the GDNF family. *J. Biol. Chem.* 272,33111-33117.

Kingsley, D.M. (1994) The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes & Development* 8,133-146.

5

Klein, R.D., Sherman, D., Ho, W.-H., Stone, D., Bennett, G.L., Moffat, B., Vandlen, R., Simmons, L., Gu, Q., Hongo, J.-A., Devaux, B., Poulsen, K., Armanini, M., Nozaki, C., Asai, N., Goddard, A.,
10 Phillips, H., Henderson, C.E., Takahashi, M. & Rosenthal, A. (1997) A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor. *Nature* 387, 717-721.

15

Kotzbauer, P.T., Lampe, P.A., Heuckeroth, R.O., Golden, J.P., Creedon, D.J., Johnson, E.M. & Milbrandt, J. (1996) Neurturin, a relative of glial-cell-line-derived neurotrophic factor. *Nature* 384, 467-470.

20

Lin, L.-F.H., Doherty, D.H., Lile, J.D., Bektesh, S. & Collins, F. (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* 260, 1130-1132.

25

Mandel, R.J., Spratt, S.K., Snyder, R.O. & Leff, S.E. (1997) Midbrain injection of recombinant adeno-associated virus encoding rat glial cell line-derived neurotrophic factor protects nigral neurons in a
30 progressive 6-hydroxydopamine-induced degeneration model of Parkinson's disease in rats. *Proc. Natl. Acad. Sci. USA* 94,14083-14088.

Marsden et al "The causes of Parkinson's disease are being unraveled and rational neuroprotective therapy is close to reality. " *Ann Neurol.* 1998 Sep;44(3 Suppl 1):S189-96

5

Masure, S., Cik, M., Pangalos, M.N., Bonaventure, P., Verhasselt, P., Lesage, A.S., Leysen, J.E. & Gordon R.D. (1998) Molecular cloning, expression and tissue distribution of glial-cell-line-derived neurotrophic factor family receptor α -3 (GFR α -3). *Eur. J. Biochem.* 251,622-630.

10

Matsushita, N., Fujita, Y., Tanaka, M., Nagatsu, T. & Kiuchi, K. (1997) Cloning and structural organization of the gene encoding the mouse glial cell line-derived neurotrophic factor, GDNF. *Gene* 203,149-157.

15

Milbrandt, J., de Sauvage, F.J., Fahrner, T.J., Baloh, R.H., Leitner, M.L., Tansey, M.G., Lampe, P.A., Heuckeroth, R.O., Kotzbauer, P.T., Simburger, K.S., Golden, J.P., Davies, J.A., Vejsada, R., Kato, A.C., Hynes, M., Sherman, D., Nishimura, M., Wang, L.C., Vandlen, R., Moffat, B., Klein, R.D., Poulsen, K., Gray, C., Garces, A., Henderson, C.E., Phillips, H.S. & Johnson, E.M. Jr. (1998) Persephin, a novel neurotrophic factor related to GDNF and neurturin. *Neuron* 20,245-253.

20

25

Moore, M.W., Klein, R.D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L.F., Ryan, A.M., Carver-Moore, K. & Rosenthal, A. (1996) Renal and neuronal abnormalities in mice lacking GDNF.

30

Nature 382, 76-79.

Mount, H.T., Dean, D.O., Alberch, J., Dreyfus, C.F. &
Black, I.B. (1995) Glial cell line-derived
5 neurotrophic factor promotes the survival and
morphologic differentiation of Purkinje cells. *Proc.*
Natl. Acad. Sci. USA 92,9092-9096.

10 Nagy Z et al "The cell division cycle and the
pathophysiology of Alzheimer's disease."
Neuroscience. 1998 Dec;87(4):731-9.

15 Naveilhan, P., Baudet, C., Mikaelis, A., Shen, L.,
Westphal, H. & Ernfors, P. (1998) Expression and
regulation of GFR α 3, a glial cell line-derived
neurotrophic factor family receptor. *Proc. Natl. Acad.*
Sci. USA 95,1295-1300.

20 Nuydens R, Dispersyn G, Van den Kieboom G, De Jong M,
Connors R, Ramaekers F, Borgers M, Geerts H "Bcl-2
protects neuronal cells against taxol-induced
apoptosis by inducing mutli-nucleation", submitted

25 Oppenheim, R.W., Houenou, L.J., Johnson, J.E., Lin,
L.F., Li, L., Lo, A.C., Newsome, A.L., Prevette, D.M.
& Wang, S. (1995) Developing motor neurons rescued
from programmed and axotomy-induced cell death by
GDNF. *Nature* 373,344-346.

30 Pichel, J.G., Shen, L., Sheng, H.Z., Granholm, A.C.,
Drago, J., Grinberg, A., Lee, E.J., Huang, S.P.,
Saarma, M., Hoffer, B.J., Sariola, H. & Westphal, H.

(1996) Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* 382, 73-76.

5 Romeo, G., Ronchetto, P., Luo, Y., Barone, V., Seri, M., Ceccherini, I., Pasini, B., Bocciardi, R., Lerone, M., Kaariainen, H. et al. (1994) Point mutations affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease. *Nature* 367,377378.

10 Sanchez, M.P., Silos-Santiago, I., Frisen, J., He, B., Lira, S.A. & Barbacid, M. (1996) Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* 382, 70-73.

15 Sanicola, M., Hession, C., Worley, D., Carmillo, P., Ehrenfels, C., Walus, L., Robinson, S., Jaworski, G., Wei, H., Tizard, R., Whitty, A., Pepinsky, R.B. & Cate, R.L. (1997) Glial cell line-derived neurotrophic factor-dependent RET activation can be mediated by two
20 different cell-surface accessory proteins. *Proc. Natl. Acad. Sci. USA* 94, 6238-6243.

Smirnova et al "Thrombin is an extracellular signal that activates intracellular death protease pathways
25 inducing apoptosis in model motor neurons". *J Neurobiol.* 1998 Jul;36(1):64-80.

Srinivisan et al "Serum from patients with type 2 diabetes with neuropathy induces complement-
30 independent, calcium-dependent apoptosis in cultured neuronal cells." *J Clin Invest.* 1998 Oct 1;102(7):1454-62

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5 Suvanto, P., Wartiovaara, K., Lindahl, M., Arumäe, U.,
Moshnyakov, M., Horelli-Kuitunen, N., Airaksinen,
M.S., Palotie, A., Sariola, H. & Saarma, M. (1997)
Cloning, mRNA distribution and chromosomal
localisation of the gene for glial cell line-derived
10 neurotrophic factor receptor β , a homologue to GDNFR α .
Human Mol. Genet. 6, 1267-1273.

Treanor, J.J.S., Goodman, L., de Sauvage, F., Stone, D.M., Poulsen, K.T., Beck, C.D., Gray, C., Armanini, M.P., Pollock, R.A., Hefti, F., Phillips, H.S., Goddard, A., Moore, M.W., Buj-Bello, A., Davies, A.M., Asai, N., Takahashi, M., Vandlen, R., Henderson, C.E. & Rosenthal, A. (1996) Characterization of a multicomponent receptor for GDNF. *Nature* 382, 80-83.

30 Functional receptor for GDNF encoded by the c-ret
proto-oncogene. *Nature* 381, 785-788.

Wellington et al "Toward understanding the molecular pathology of Huntington's disease. "Brain Pathol. 1997 Jul;7(3):979-1002.

- 5 Widenfalk, J., Nosrat, C., Tomac, A., Westphal, H.,
Hoffer, B. & Olson, L. (1997) Neurturin and glial cell
line-derived neurotrophic factor receptor- β (GDNFR- α),
novel proteins related to GDNF and GDNFR- α with
specific cellular patterns of expression suggesting
10 roles in the developing and adult nervous system and
in peripheral organs. *J. Neurosci.* 17,8506-8519.

- Widenfalk, J., Tomac, A., Lindqvist, E., Hoffer, B. &
Olson, L. (1998) GFR α -3, a protein related to GFR α -1,
15 is expressed in developing peripheral neurons and
ensheathing cells. *Eur. J. Neurosci.* 10,1508-1517.

- Worby, C.A., Vega, Q.C., Zhao, Y., Chao, H. H.-J.,
Seasholtz, A.F. & Dixon, J.E. (1996) Glial cell line
20 derived neurotrophic factor signals through the RET
receptor and activates nitogen-activated protein
kinase. *J. Biol. Chem.* 271, 23619-23622.

- Worby, C.A., Vega, Q.C., Chao, H.H.J., Seasholtz,
25 A.F., Thompson, R.C. & Dixon J.E. (1998)
Identification and characterization of GFR α -3, a novel
co-receptor belonging to the glial cell line-derived
neurotrophic receptor family. *J. Biol. Chem.* 273,3502-
3508.

- 30 Yan, Q., Matheson, C. & Lopez, O.T. (1995) In vivo
neurotrophic effects of GDNF on neonatal and adult
facial motor neurons. *Nature* 373,341-344.

List of abbreviations

	BLAST	basic local alignment search tool
	bp	base pairs
5	cDNA	complementary DNA
	CNS	central nervous system
	EST	expressed sequence tag
	EVN	enovin
	GDNF	glial cell-line derived
10		neurotrophic factor
	GFR α	GDNF family receptor α
	GPI	glycosyl phosphatidyl inositol
	MTC	multiple tissue cDNA
	NTN	neurturin
15	PCR	polymerase chain reaction
	PNS	peripheral nervous system
	PSP	persephin
	RT-PCR	reverse transcription PCR
	TGF- β	transforming growth factor β
20	FISH	fluorescent <i>in situ</i> hybridisation
	MTN	multiple tissue northern
	NGF	nerve growth factor
	SPR	surface plasmon resonance